*Biochimica et Biophysica Acta*, 775 (1984) 374–380
Elsevier

BBA 72237

# THE DYNAMICS OF LIPID MOTION IN SARCOPLASMIC RETICULUM MEMBRANES DETERMINED BY STEADY-STATE AND TIME-RESOLVED FLUORESCENCE MEASUREMENTS ON 1,6-DIPHENYL-1,3,5-HEXATRIENE AND RELATED MOLECULES

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(Received March 5th, 1984)

*Key words: Lipid dynamics; Sarcoplasmic reticulum; Fluorescence probe; Steady-state fluorescence; Time-resolved fluorescence; Fluorescence; (Rabbit skeletal muscle)*

**Steady-state and time-resolved fluorescence anisotropy measurements were made on 1,6-diphenyl-1,3,5-hexatriene (DPH), 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) and 1-acyl-2-(DPH)-phosphatidylcholine (DPH-PC) incorporated into sarcoplasmic reticulum membranes. The results were analysed in terms of the 'wobbling-in-cone' model. Considerable differences in the fluorescence parameters were found. In particular TMA-DPH and DPH-PC showed a smaller cone angle, relating to the range of acyl chain motion, compared to DPH, taken to be a reflection of a difference in probe locations. The influence of the protein component was also found to restrict DPH motion more than TMA-DPH and DPH-PC. Effectiveness in assessment of perturbation of the membrane by the non-esterified fatty acid, oleic acid again revealed differences. The steady-state anisotropy decreased on addition of oleic acid; a recovery to control values was observed with DPH but not with the other probes. Time-resolved parameters followed the same pattern. The results of this work demonstrated the effectiveness of these three probes in revealing differences in membrane properties, such as protein and fatty acid perturbation of membrane lipid structure and dynamics.**

## Introduction

The fluorescence probe DPH has been widely used to monitor the motional characteristics of the hydrocarbon region of membranes and its response to many other membrane and non-membrane components. Analysis of time-resolved anisotropy decays, according to the 'wobbling-in-cone' model, provides simultaneous measurement of parameters relating to both the range of acyl chain motion (cone angle, $\theta_c$) and the rate of motion (wobbling diffusion constant, $D_w$). For DPH these have been determined for simple model systems [1–5] including studies on the effect of cholesterol [4,6] and in natural [7] and reconstituted membranes [8], where the effect of the protein component was assessed. The precise location of DPH in the membrane lipid bilayer is rather uncertain so that information regarding regions of different depths within the bilayer cannot

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Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene; DPH-PC, 1-acyl-2-(DPH)-phosphatidylcholine.

be obtained. Recently a number of new DPH type molecules have been synthesized [9–12], many of which include a charged species which, in the case of TMA-DPH, has been suggested to effectively locate the DPH more towards the lipid head-group region [13].

In this work the time-resolved and steady-state fluorescence parameters of DPH, TMA-DPH and DPH-PC were compared in the sarcoplasmic reticulum membrane system with respect to the influence of protein and non-esterified fatty acids on lipid motion.

## Materials and Methods

*Chemicals.* DPH and TMA-DPH were obtained from Molecular Probes. DPH-PC, prepared from egg yolk lysophosphatidylcholine acylated with 4-(2′-carboxylethyl)-DPH, synthesized as previously described [12], was kindly provided by Professor R.B. Cundall. The probes were dissolved in tetrahydrofuran (DPH) or ethanol (TMA-DPH, DPH-PC). Oleic, linoleic and palmitic acids were from Sigma (U.K.). All chemicals were of analar grade.

*Membrane and liposome preparations.* Rabbit hind leg white skeletal muscle sarcoplasmic reticulum membranes were prepared according to Warren et al. [14]. Purity of the preparation was checked by gel-electrophoresis, transmission electron microscopy, and specific activity of $Ca^{2+}$-ATPase and was found to consist almost entirely of vesicles of sarcoplasmic reticulum. Multilamellar liposomes were prepared from a lipid extract of the membranes in chloroform obtained according to the method of Bligh and Dyer [15]. The chloroform was removed by evaporation under reduced pressure, addition of phosphate buffer (0.05 M, pH 7.4) and vigorous vortexing.

*Incorporation of probes into membranes.* Intact membranes were labelled with the DPH probes by addition of the required amount of probe in solvent to a suspension of the membranes in buffer with vigorous stirring, followed by a period of 30 min before measurements were made. The probes were incorporated into the lipid liposomes by adding the required amount to a chloroform solution of the membrane lipids. The solvents were removed and liposomes made as above.

*Addition of fatty acids.* In most of the experiments the fatty acids, in ethanol, were added directly to intact membranes. Fatty acids were also directly introduced into the membranes by first evaporating the ethanol (in the measuring cuvette) followed by addition of the membrane suspension and stirring and immediate monitoring of the steady-state anisotropy. The extent of incorporation of the fatty acids into membranes was assessed by addition of 2 $\mu$Ci of $^3$[H]oleic acid (New England Nuclear), followed by centrifugation at $100\,000 \times g$ and determination of the radioactivity in the pellet (membranes and supernatant were dissolved in a suitable scintillation cocktail) using an Aloka scintillation counter.

*Fluorescence measurements.* Decays of the fluorescence anisotropy and total fluorescence intensity were measured using a single photon counting fluorimeter as previously described [16] and subsequently modified [7]. The excitation light (using a xenon lamp in place of the $H_2$ lamp for steady-state measurements) was passed through a band pass monochromator (360 nm), Glan prism polarizer and a Hoya U-340 filter. Emission was passed through Fuji-Film SC-39 and Hoya L-42 filters (transmission above 420 nm) and polaroid HNB′B sheet polarizers.

*Analysis of fluorescence data.* Steady-state fluorescence anisotropy ($r^s$) was calculated according to the relationship:

$$r^s = \frac{I_v + I_h}{I_v - 2I_h} \tag{1}$$

where $I$ refers to the fluorescence intensity through polarizers oriented vertically (v) and horizontally (h) with respect to the plane of polarization of the excitation beam. The difference in the sensitivities of the detection system for vertically and horizontally polarized light was cancelled by polarization scramblers. The necessity for making corrections due to scattering was avoided by using sufficiently dilute samples.

The nanosecond decay data were analysed by assuming exponential decays of the following forms:

$$I_T^\delta(t) = \alpha_1 \exp(-t/\tau_1) + \alpha_2 \exp(-t/\tau_2) \tag{2}$$

$$r^\delta(t) = (r_0 - r_\infty) \exp(-t/\phi) + r_\infty \tag{3}$$

where the superscript $\delta$ indicates that these quantities are responses to impulsive ($\delta$-like function) excitations. The parameters $\alpha_{1,2}$ (decay amplitudes), $\tau_{1,2}$ (fluorescence lifetimes), $r_\infty$ (limiting anisotropy), $\phi$ (apparent relaxation time) were so determined that the convoluted products $g(t) * I_T^\delta(t)$ and $g(t) * [I_T^\delta(t) \cdot r^\delta(t)]$, best fitted the observed $I_T(t)$ and $I_D(t)$, respectively [16]. The fundamental anisotropy, $r_0$, was taken to be 0.395. This was previously shown for DPH [3] and TMA-DPH has an identical $r_0$ value [13] while the assumption for DPH-PC remains to be verified. The alternative method for determining $r_\infty$, of leaving $r_0$ as a free parameter, resulted in only slightly differing $r_\infty$ values, but lower $r_0$ values. The cause of the lower $r_0$ values is due to the interference of the finite excitation pulse and we feel that the best solution at present is to fix $r_0$, this however reduces the accuracy of the $\phi$ values which are obtained from the initial $r(t)$ decay. Calculations were carried out on a LSI-1 or Facom M-300 computer. The cone angle, $\theta_c$, was calculated from $r_\infty$ using the relationship:

$$r_\infty / r_0 = \cos^2\theta_c (1 + \cos\theta_c)^2 / 4 \qquad (4)$$

The wobbling diffusion constant, $D_w$, was estimated from the following equation, where $x = \cos\theta_c$ [17], which is equivalent to the original numerical result [18]:

$$\begin{aligned} D_w \phi (r_0 - r_\infty)/r_0 = &-x^2(1+x^2)\left[\ln[(1+x)/2]\right. \\ &+ (1-x)/2\left.\right]/[2(1-x)] \\ &+ (1-x)(6 + 8x - x^2 \\ &- 12x^3 - 7x^4)/24 \end{aligned} \qquad (5)$$

*Chemical analyses.* The protein and phospholipid content of the membrane preparations were determined according to the methods of Lowry et al. [19] and Bartlett [20], respectively.

## Results and Discussion

*Incorporation of probes*

The incorporation of DPH and TMA-DPH into the membranes was indicated by an increase in fluorescence intensity as the probes moved from the aqueous phase to the hydrophobic domain of the membrane lipid. In contrast DPH-PC alone was fluorescent, presumably because it has a low critical micelle concentration and tends to form micelles of an undetermined character. It was therefore necessary to remove the DPH-PC which had not incorporated into the membrane after a suitable incubation period (30 min). The proportion of fluorescence intensity which pelleted at $100\,000 \times g$, presumably representing probe associated with the membrane (Table I), was lower for DPH-PC confirming the slower rate of uptake.

*Time-resolved fluorescence measurements*

The time-resolved fluorescence parameters for the DPH probes in intact sarcoplasmic reticulum membranes and liposomes prepared from total lipid extracts of the membrane are shown in Table II. The decay of the fluorescence intensity of DPH

TABLE I

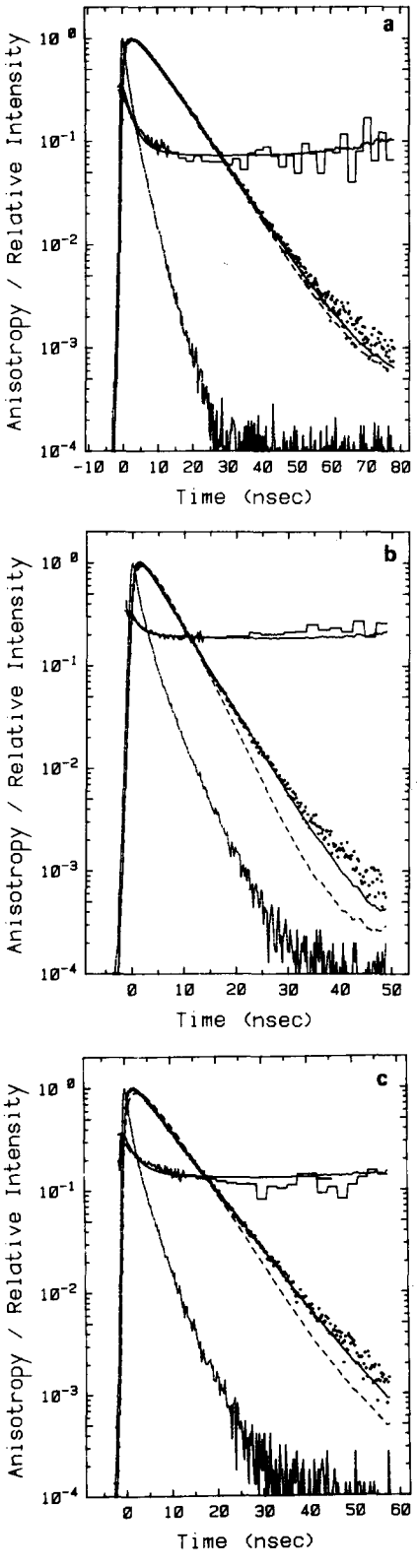
DISTRIBUTION OF DPH, TMA-DPH, DPH-PC AND 18:1 IN INTACT SARCOPLASMIC RETICULUM MEMBRANES AND SOLUBLE FRACTIONS AFTER CENTRIFUGATION AT $100\,000 \times g$

After centrifugation at $100\,000 \times g$ the proportion of fluorescence intensity or $^3$H recovered in the membrane pellet was determined.

| Addition ($\mu$mol 18:1/mg protein) | Centrifugation (min) | Probe | % fluorescence intensity or $^3$H radioactivity in membranes |
|---|---|---|---|
| – | 10 | DPH | 84.6 |
| – | 30 | DPH | 93.0 |
| 2 | 10 | DPH | 87.3 |
| 2 | 10 | TMA-DPH | 82.7 |
| 2 | 10 | DPH-PC | 66.8 |
| 2 | 10 [a] | DPH | 86.0 |
| 4 | 10 | DPH | 86.0 |
| 2 | 30 | DPH | 99.0 |
| – | 10 [b] | DPH | 71.8 |
| 2 | 10 [b] | DPH | 85.0 |
| 2 | 10 | $^3$[H]18:1 | 61.6 |
| 2 | 30 | $^3$[H]18:1 | 66.9 |

[a] Centrifugation 2 h after 18:1 addition (60 $\mu$M, 0.03 mg protein/ml).

[b] Pellet was resuspended and re-centrifuged for a further 10 min.




(Fig. 1A) although reasonably described by a single exponential decay process was nevertheless routinely analysed as a double exponential decay. With TMA-DPH and DPH-PC, description of the fluorescence decay as mono-exponential is clearly inadequate (Figs. 1B, C). Again the results were analysed as double exponential decays, although an additional long third component gave a slightly improved fit to the data.

The presence of the major short lifetime component, shown by the size of the decay amplitudes, for DPH-PC and TMA-DPH has some consequence on the analysis of the fluorescence anisotropy decay data in terms of $\theta_c$ and $D_w$. Firstly the contribution to $r(t)$ from the short lived component decreases rapidly, while $r_\infty$ or $\theta_c$, by definition, refer only to the long lived component. Also $D_w$, as found by the approximation of Eqn. 3 above, may partially reflect a decreasing contribution from short lived components. This uncertainty in the time-resolved fluoresce anisotropy measurements revealed in this study must await further investigation.

The most important and striking difference between the probes is the much smaller value of $\theta_c$ for TMA-DPH and DPH-PC as compared to DPH. The order in increasing $\theta_c$, for both intact membranes and lipid liposomes was TMA-DPH < DPH-PC < DPH (Table II). The smaller $\theta_c$ is indicative of a more restricted motional environment. By virtue of the charge on TMA-DPH it would be expected that the molecule would be oriented with tee charge located at the head-group region [13], adjacent to the C1-C10 region of the membrane phospholipid fatty acyl chains. This would account for the more restricted motion and comparison may be made between $^2$H-NMR studies showing a higher order parameter (equivalent to a smaller

Fig. 1. Fluorescence decays for DPH (A), TMA-DPH (B) and DPH-PC (C) in intact membranes at 37°C. Chain line, the instrumental response function, $g(t)$; dots, the total fluorescence intensity, $I_T(t)$; zigzag solid curves, fluorescence anisotropy, $r(t)$. The broken and solid lines superimposed on $I_T(t)$ are the calculated best-fit curves for single and double exponential approximations, respectively. The smooth line superimposed on the $r(t)$ curve is the calculated best-fit curve according to Eqn. 3. Experimental details were as described in Materials and Methods.

TABLE II

FLUORESCENCE PARAMETERS OF DPH, DPH-PC AND TMA-DPH IN INTACT SARCOPLASMIC RETICULUM MEMBRANES AND LIPOSOMES MADE FROM TOTAL LIPID EXTRACTS OF THE MEMBRANES AT 37 °C

$r^s$, steady-state anisotropy; $r_\infty$, residual equilibrium anisotropy; $\theta_c$, cone angle; $\phi$, apparent relaxation time; $D_w$, wobbling diffusion constant; $\tau_{1,2}$ and $\alpha_{1,2}$, fluorescence lifetimes under double exponential approximation and respective decay amplitudes.

| | | $r^s$ | $r_\infty$ | $\theta_c$ (deg) | $\phi$ (ns) | $D_w$ | $\alpha_1$ | $\tau_1$ | $\alpha_2$ | $\tau_2$ |
|---|---|---|---|---|---|---|---|---|---|---|
| Intact membranes | | | | | | | | | | |
| DPH | control | 0.122 ± 0.001(9) [a] | 0.071 | 56.7 | 1.33 | 0.151 | 0.26 | 1.54 | 0.74 | 8.67 |
| | +18:1 [b,c] | 0.096 | 0.052 | 60.8 | 1.19 | 0.182 | 0.28 | 1.53 | 0.72 | 8.60 |
| | +18:1 [d] | 0.111 | 0.063 | 58.3 | 1.21 | 0.171 | 0.29 | 1.68 | 0.71 | 8.56 |
| | +16:0 | 0.113 | 0.073 | 56.3 | 1.32 | 0.151 | 0.25 | 1.80 | 0.75 | 8.70 |
| DPH-PC | control [e] | 0.187 ± 0.004(3) [a] | 0.126 | 47.6 | 1.12 | 0.145 | 0.63 | 1.48 | 0.37 | 6.96 |
| | +18:1 [e] | 0.171 ± 0.004(3) [a] | 0.108 | 50.3 | 1.14 | 0.151 | 0.55 | 1.53 | 0.45 | 7.00 |
| TMA-DPH | control | 0.238 ± 0.001(3) [a] | 0.182 | 39.8 | 0.57 | 0.209 | 0.69 | 1.51 | 0.31 | 5.14 |
| | 18:1 | 0.215 ± 0.003(3) [a] | 0.157 | 43.2 | 0.60 | 0.227 | 0.72 | 1.40 | 0.28 | 4.43 |
| Membrane lipid liposomes | | | | | | | | | | |
| DPH [f] | | 0.071 | 0.021 | 70.0 | 0.99 | 0.248 | 0.27 | 1.18 | 0.73 | 8.14 |
| DPH – PC [f] | | 0.166 | 0.101 | 51.6 | 1.05 | 0.170 | 0.58 | 1.53 | 0.43 | 6.02 |
| TMA-DPH [f] | | 0.183 | 0.125 | 47.8 | 0.79 | 0.215 | 0.65 | 1.30 | 0.22 | 4.65 |

[a] Data ± standard deviation, number of results in brackets.
[b] Data collection time, first 10 min after 18:1 addition (60 μM, 0.03 mg protein/ml).
[c] Fluorescence decay data of four experiments added: combined data subject to analysis.
[d] Data collection time, 10 min commencing 60 min after 18:1 addition.
[e] Time-resolved fluorescence data, mean of three separate experiments.
[f] Mean of two experiments, one of which was a combination of the decay data of three separate experiments.

cone angle) as one examines regions more towards the lipid head-group region of the bilayer [21]. The value for DPH-PC falls between the values for the other probes as might be expected. It is also likely that the locations of the DPH probes are such that the regions sensed overlap.

Comparison of the time-resolved fluorescence parameters between intact membranes and liposomes derived from the lipids thereof revealed further differences (Table II). While all probes showed an increase in motional freedom in the absence of protein ($\theta_c$ increase), the effect was much greater for DPH. The increases in $\theta_c$ were 14.3, 8.0, and 4.0 for DPH, TMA-DPH and DPH-PC, respectively. It may be that DPH being untethered is able to locate in regions on or in clefts within the proteins, or between protein molecules where there would be considerable constraint on probe motion. If these regions were at the centre of the bilayer they might be expected to be inaccessible to TMA-DPH and DPH-PC.

*Addition of fatty acids*

The effectiveness of the three probes in assessing perturbation by membrane 'fluidizing' agents was compared by examining the effects of additions of non-esterified fatty acids. The interaction of fatty acids with membranes is of interest both for use as a model for studying the effects of drugs on membranes and since they are released into membranes from endogenous phospholipids on interaction of cells with various external stimuli. In this work we were concerned with the usefulness of the DPH probes in examining the gross disturbance of the membrane which would be caused by the relatively high levels of oleic acid added. This might have physiological significance regarding the high levels which would be found around phospholipases, for instance, as they release the fatty acids into the membrane.

The effect of oleic acid on the $r^s$ of DPH in membranes at 37°C is shown in Fig. 2. At this level (60 μM, 0.03 mg protein/ml) it has been shown that $Ca^{2+}$ uptake is inhibited and the ATPase activity (uncoupled from the $Ca^{2+}$ up-

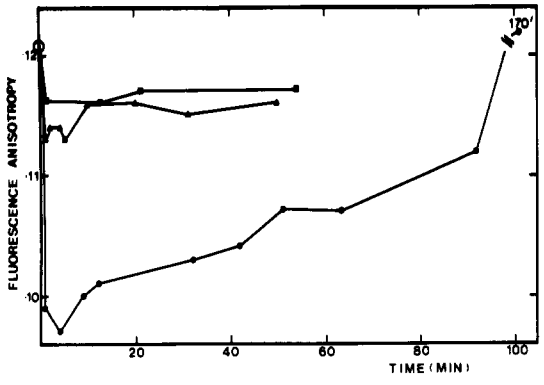



Fig. 2. Change in the steady-state anisotropy of DPH with time in intact membranes at 37°C: control, 8.7 μM ethanol, 0.03 mg protein/ml (■); 16:0 (▲) and 18:1 (●) 60 μM (in ethanol). Experimental details were as described in Materials and Methods.

take) is inhibited by 50–75% [22–24]. In contrast to the effect of the unsaturated oleic acid, the saturated palmitic acid had little effect on the DPH $r^s$ values. After the initial rapid decrease in $r^s$ there was a gradual recovery to control levels. At higher concentrations of oleic acid (Fig. 3) the anisotropy did not fully recover indicating that the membrane had been reversibly disturbed. The di-unsaturated linoleic acid had a similar effect (results not shown). Decreasing the temperature to 4°C had no effect on the kinetics of the changes in $r^s$. These results can be compared to those of the effects of fatty acids on lymphocyte membranes where a similar effect on $r^s$ was found [25,26], however, a time-dependent recovery in the $r^s$ values was not apparently found. In Fig. 4 the effect of oleic acid on the $r^s$ values of all three probes, on both intact membranes and derived total lipid liposomes is shown. Although all probes showed a similar rapid decrease in $r^s$, only with DPH was there a full recovery to control values. When the effects of fatty acid addition on the time-resolved fluorescence parameters where examined these were found to reflect the same time dependent effects (on $\theta_c$ and $D_w$) as shown in Table II.

The effects of ethanol, in which the oleic acid

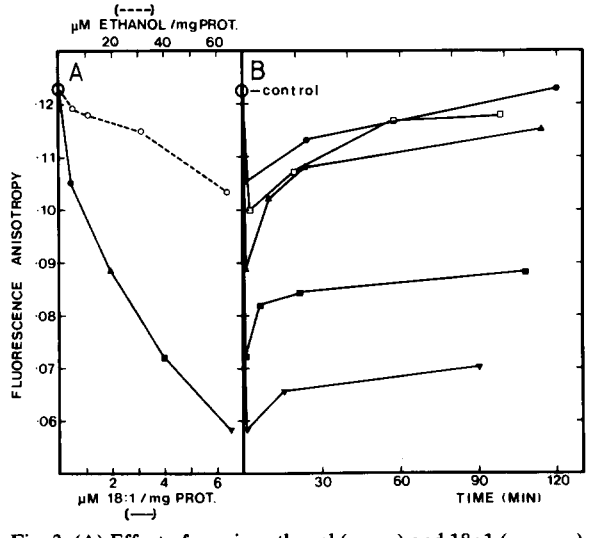



Fig. 3. (A) Effect of varying ethanol (– – –) and 18:1 (———) concentration on the steady-state fluorescence intensity of DPH 1 min after addition to intact membranes at 37°C. (B) Change in the steady-state fluorescence anisotropy of DPH with time after addition of 18:1 added in ethanol (●, 1.0; ▲, 2.0; ■, 4.0; ▼ 6.5 μmol 18:1/mg protein) and without ethanol (□, 2.0 μmol 18:1/mg protein) at 37°C. Experimental details were as described in Materials and Methods.

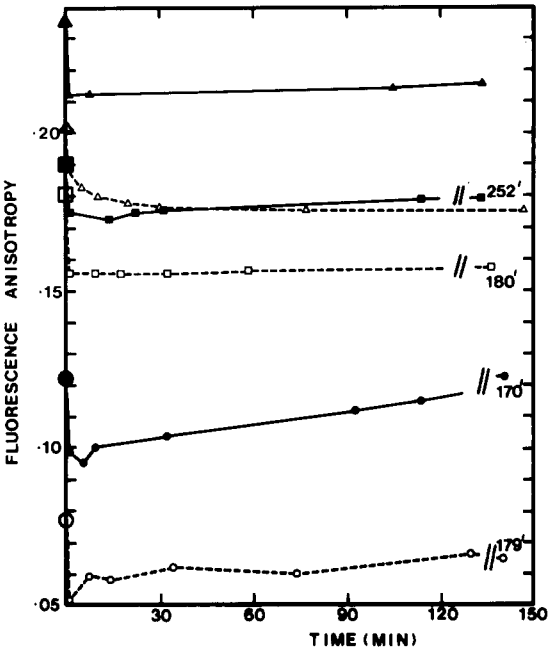



Fig. 4. Change in the steady-state anisotropy of DPH (●, ○), TMA-DPH (▲, △) and DPH-PC (■, □) in intact membranes (solid symbols) and membrane lipid liposomes (open symbols) with time, on addition of 18:1 (60 μM, 0.03 μ protein/ml) at 37°C. The larger symbols (zero time) indicate the control anisotropy values. Experimental details were as described in Materials and Methods.

was added, were also examined to verify that at the low levels added no effect on the membrane was occurring (Fig. 3). Thus at the highest concentration used (20 $\mu$mol/mg protein) it can be seen that any effect on $r^s$ values would have been insignificant. This was checked by examining the effect of addition of oleic acid without ethanol as described in Materials and Methods. Again a similar effect on $r^s$ with time was found (Fig. 3).

In order to examine the fatty acid incorporation into the membrane the effect of oleic acid on the distribution of the probes in the intact membranes (100000 $\times$ $g$ pellet) and in the soluble fraction (100000 $\times$ $g$ supernatant) was determined (Table I). The $r^s$ values for the supernatant were similar to those for the membrane fraction which indicates that the soluble material was of a membranous nature and not purely of lipid origin or non-incorporated oleic acid, in which case the $r^s$ value would have been much lower; confirmation of this will have to await further analysis. Furthermore the same time-dependent changes in $r^s$ were found for all probes in both fractions. The results also show that oleic acid did not effect the distribution of the probes between the soluble and membrane fractions. This was the case whether centrifugation was performed immediately after oleic acid addition or after 2 h, when the $r^s$ for DPH had recovered. The incorporation of oleic acid was determined by addition of $^3$[H]oleic acid followed by centrifugation as before. The results (Table I) show that around 60% was incorporated into the membrane.

In conclusion the most striking result of the effect of oleic acid perturbation was the lack of recovery of regions mointored by TMA-DPH and DPH-PC compared to DPH. This would indicate that the probes are reporting on differing locations in the perturbed sarcoplasmic reticulum membrane. More studies are required to fully characterize these effects, however, the usefulness of the three DPH probes in assessing different regions of membranes has been demonstrated.

## Acknowledgements

We gratefully acknowledge financial support in the form of special coordination funds for promoting science and technology and a grant for 'Solar Energy-Photosynthesis' from the Agency of Science and Technology of Japan, the SERC (U.K.), the Royal Society and Professor R.B. Cundall for the gift of DPH-PC.

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