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PHOTOCHEMICAL CHANGES OF FLUORESCENT PROBES IN MEMBRANES AND THEIR EFFECT ON THE OBSERVED FLUORESCENCE ANISOTROPY VALUES

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The fluorescence intensity of diphenylhexatriene (DPH) and of trimethylammonium-diphenylhexatriene (TMA-DPH) is measured when these probes are embedded in vesicles of dipalmitoyl- and dioleoylphosphatidylcholine (DPPC and DOPC), in mixtures of these vesicles as well as in vesicles of the mixed phospholipids, in trout intestinal brush border membranes and in mitoplasts of rat liver cells. The intensity in DOPC vesicles is found to be significantly higher than in DPPC vesicles. When these systems are irradiated with strong ultraviolet light radiation, a decrease in the fluorescence intensity is observed; this effect is much stronger in DOPC than in DPPC vesicles. The fluorescence anisotropy values in the mixture of vesicles as well as in the membranes show an initial increase with irradiation which is followed by a significant decrease. A transfer of DPH molecules between DPPC and DOPC vesicles is observed. For TMA-DPH this transfer takes place only from DPPC to DOPC vesicles, but not vice-versa. These results are related to intensity and anisotropy measurements of these probes in cell cultures.

Introduction

With the introduction of the use of DPH as a fluorescent probe for cell membrane studies a (reversible) effect of photoisomerization was observed which caused a reduction of the fluorescence intensity of this molecule [1]. For measurements of cell suspensions this effect is practically negligible because of the continuous movement of the irradiated particles out of the light beam and the return of the molecules to their normal (fluorescent) state. For measurements in cell cultures, however, in which the cells are fixed and hence the same cells are continuously exposed to the exciting radiation, significant changes in the intensity can be observed, even for rather short periods of excitation, depending, of course, on the intensity of the radiation. In an extended investigation of melanoma cell cultures we found that the decrease in intensity of DPH, due to this effect is accompanied by a significant increase in the anisotropy (or polarization) values of the fluorescence [2]. We explained this increase in the anisotropy value in the following way: it is now rather well established that DPH is distributed over all lipidic components of the cell [3,4]. If the photoisomerization of DPH into a non-fluorescent state increases with decreasing viscosity (due to enhanced rotational freedom) then the fluorescence will ultimately be destroyed in the more fluid parts of the cell and only the molecules in the most viscous parts will remain intact, and hence the observed anisotropy

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Abbreviations: DPH, diphenylhexatriene; TMA-DPH, trimethylammonium-diphenylhexatriene.

will increase. This interpretation was supported by our observation that at low temperatures the radiation effect is practically eliminated due to the increased viscosity of all parts of the cell. The increase in the values with time of measurement which is observed at higher temperatures is limited not only by the anisotropy values of the most viscous part of the cell but also by the reverse process which spontaneously reestablishes the fluorescent state of a part of the molecules and which is again faster if the viscosity is low.

Another marker which we used in our anisotropy measurements on cell cultures was trimethylammonium-diphenylhexatriene (TMA-DPH). This is a rather novel marker; its properties have been described by Prendergast et al. [5]. This molecule is believed to remain in the plasma membrane [6], since its polar head is most probably anchored at the lipid-water interface while the hydrocarbon moiety (DPH) enters the lipid part of the membrane. According to the above interpretation the anisotropy of TMA-DPH should not increase with irradiation time, since it does not enter different regions of the cell. This was indeed found to be so. Actually in many cell cultures a decrease in the anisotropy values of TMA-DPH with time of measurement was found. We shall see that such effects are also observed for TMA-DPH in suspensions of membranes.

It is clear that a correct evaluation of our polarization studies in cell cultures depends rather critically on the soundness of these assumptions. Hence we decided to study the photochemical effect on these markers when they are embedded in various lipidic systems. The results seem to correlate well with the behaviour of these markers in cells.

Materials and Methods

Vesicles

Dipalmitoyl- and dioleoylphosphatidylcholine (DPPC and DOPC) were purchased from Sigma Chemical Co. and used without further purification. Weighed samples of phospholipids were dissolved in chloroform; the solvent was then evaporated with a stream of dry nitrogen which led to the deposition of the lipids on the walls of the vessel. The lipids were then dispersed in water by a cyclo-mixer (Clay-Adams) at temperatures above their phase transition ranges. Unilamellar vesicles were prepared by sonication with a bath type sonicator (Bandelin Sonorex RK 102) according to Lawaczek et al. [7] and Szoka and Papahadjopoulos [8]. The resulting solutions were diluted in order to have a turbidity at 350 nm (excitation wavelength) of 0.1 absorbance units, corresponding to concentration of about 0.19 mg/ml and 0.13 mg/ml for DPPC and DOPC, respectively. Such a low turbidity precludes any correction for light-scattering in fluorescence anisotropy measurements [9].

Biological membranes

Intestinal brush border membranes of trout were a gift of Dr. Leray (Laboratoire de Physiologie Comparée des Régulations du CNRS, Strasbourg), and were prepared according to Schmitz et al. [10] as modified by Hauser et al. [11]. Experiments were done in 2 mM Tris-HCl buffer, pH 7, 50 mM mannitol. Mitoplasts from rat-liver mitochondria (i.e. the submitochondrial fraction that remained after the removal of the external membrane) were a gift of Dr. Cremel (Centre de Neurochimie du CNRS, Strasbourg) and were prepared according to Lévy et al. [12] as modified by Waksman and Rendon [13]. Experiments were done in 5 mM Hepes-HCl buffer, pH 7, 0.24 M sucrose.

Fluorescent probes and labeling

Diphenylhexatriene (DPH) from Koch-Light Laboratories and trimethylammonium-DPH (TMA-DPH) from Molecular Probes were stored in stock solutions (in tetrahydrofuran and dimethylformamide of fluorescence grade, respectively) at a concentration of 10^{-3} M. The basic procedure for labeling samples has been described previously [14]. The final concentrations of the probes were $0.33 \cdot 10^{-6}$ M, giving a ratio of label/phospholipid of about 1/500 for the vesicles and about 1/200 for the biological membranes.

Experimental

Absorbance measurements were performed on a Cary 219 spectrophotometer.

Fluorescence anisotropy measurements were

performed on a SLM 8000-Sc spectrofluorometer, giving a precision of a least 10^{-3} anisotropy units, for the lowest fluorescence intensities measured. The excitation wavelength was 350 nm, and the emission wavelength 425 nm (the latter was selected by Schott interference filters). Samples were placed into a temperature controlled water jacket, with a platinum resistance probe directly immersed into the cuvette, thus permitting an accuracy of 0.1 deg. C. When not stated otherwise, intensity and polarization measurements were performed at 20°C.

For the study of the photochemical changes in the vesicles and membranes containing DPH or TMA-DPH, the suspensions were irradiated by radiation of 365 nm wavelength from a Schoeffel irradiation unit consisting of a 100 W Xe-Hg mercury lamp and a high intensity monochromator. The intensity at the front window of the cell was roughly 6 μW.

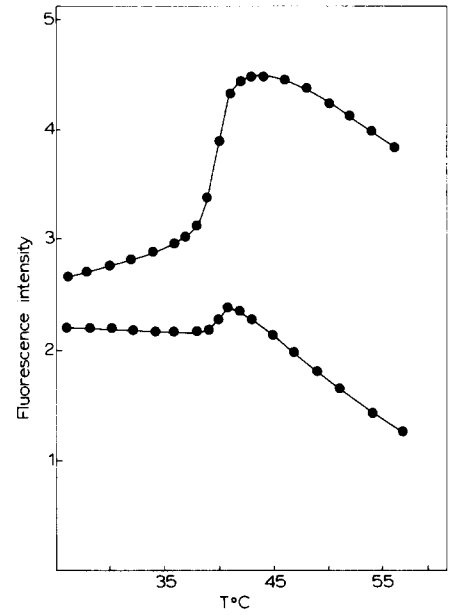

Fig. 1. Temperature dependence of fluorescence intensity for DPH (upper curve) and TMA-DPH (lower curve) embedded in DPPC vesicles.

Results and Discussion

(1) Dependence of fluorescence intensity of DPH and TMA-DPH on the viscosity of the medium

The fluorescence intensity of DPH and of TMA-DPH in DOPC vesicles was found to be significantly higher than in DPPC vesicles (for equal concentrations). The ratio of intensities varied between 1.5 and 4. Because DPPC vesicles are in a gel phase at 20°C and DOPC vesicles are liquid crystalline, we tend to ascribe the decrease in intensity in the more viscous medium to a torsional effect of the lipid chains on the embedded marker molecule, which perturbs its symmetry and enhances non-radiative transitions. As seen in Fig. 1, this assumption gains support from the increase of fluorescence intensity of DPH or TMA-DPH embedded in DPPC vesicles when the temperature raises from 38°C to 42°C, corresponding to DPPC phase transition.

Fluorescence lifetime datas for the probes embedded in DPPC and DOPC vesicles are summarized in Table I, and were obtained from Stubbs et al. [15] and from Prendergast et al. [5] for DPH and TMA-DPH, respectively. A shorter lifetime always corresponds to a greater fluidity (obtained by increasing temperature). Moreover, we can notice that for both probes, lifetimes are quite identical in DPPC vesicles at 50°C and in DOPC vesicles at 25°C, the fluidity of which, measured by r_{DPH}, are the same [15].

(2) Dependence of photoisomerization of DPH and of TMA-DPH on the viscosity of the medium

The photochemical conversion of DPH and TMA-DPH in solutions or suspensions is not observed for intensities which are normally used in polarization measurements. Hence in order to demonstrate this effect (which is clearly observed in cell cultures, see Introduction) we irradiated the

TABLE I

FLUORESCENCE LIFETIMES FROM DPH AND TMA-DPH EMBEDDED IN DPPC AND DOPC VESICLES

	Temp. (°C)	Fluorescence lifetime (ns)	
		DPH (Ref. 15)	TMA-DPH (Ref. 5)
DPPC	25	9.5	6.3
	50	8.0	3.4
DOPC	25	7.7	3.5
	50	7.1	2.8

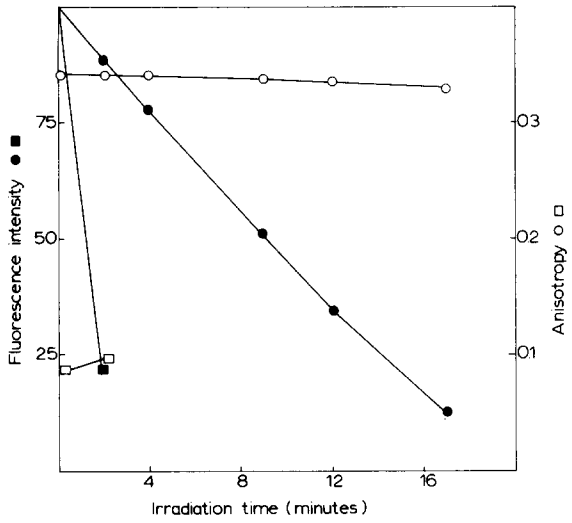

Fig. 2. Variation of fluorescence intensity and anisotropy of DPH embedded in DPPC (circles) and DOPC (squares) vesicles with time of irradiation. Intensities of non-irradiated samples normalized to 100.

suspensions in the manner indicated in Materials and Methods.

Fig. 2 shows the dependence on time of irradiation of the fluorescence intensity of DPH in DPPC and in DOPC vesicles. It is seen that the effect is much stronger in the medium of lower viscosity.

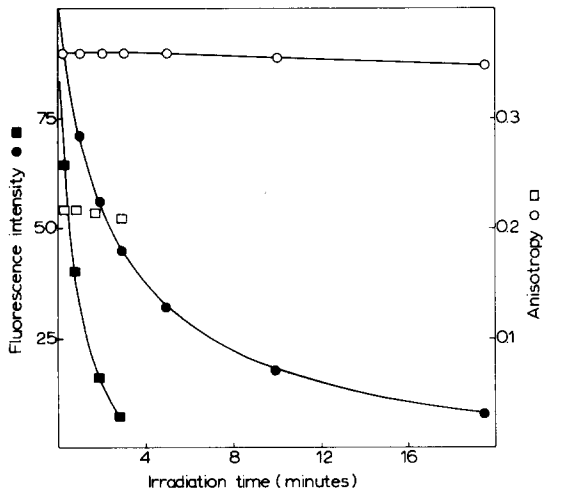

Fig. 3. Variation of fluorescence intensity and anisotropy of TMA-DPH embedded in DPPC and DOPC vesicles with time of irradiation. Symbols as for Fig. 2.

This is expected if the underlying mechanism is photoisomerization (as suggested above), since this mechanism would be more effective in a medium which permits greater rotational freedom. This agrees also with the distortion effect on the probe by the more viscous medium, as suggested above, which leads to a decrease in intensity.

Fig. 3 shows the dependence on time of irradiation of the fluorescence intensity of TMA-DPH in DPPC and in DOPC vesicles. Again we see the much faster decrease in the fluorescence yield of the marker in the more fluid medium.

These results strongly support our view that the apparent increase in the anisotropy values of DPH with time of measurement which we observed in cell cultures is due to the faster photo-deactivation of the marker in the more fluid parts of the cell. On the other hand they lend support to the assumption that TMA-DPH is placed predominantly in one lipidic system of the cell, most probably the plasma membrane [5,6] since with this probe no increase in the polarization values in cell cultures was found. The direct proof of this latter assumption may meet very great difficulties as we will see in the following.

Almost exceptionless we found for all systems an increase in fluorescence intensity with time after cessation of the irradiation, indicating the return of a part of the molecules to their normal (fluorescent) state.

(3) Dependence of the fluorescence anisotropy of DPH and of TMA-DPH in DPPC and DOPC vesicles on time of irradiation

The anisotropy values for the non-irradiated samples (at 20°C) are 0.329 and 0.088 for DPH embedded in DPPC and DOPC vesicles, respectively, and 0.258 and 0.218 for TMA-DPH in the same vesicles.

The values for DPH agree well with those given by Stubbs et al. [15]. Irradiation of the samples has only a slight influence on the anisotropy values. After drastic reduction of the intensity, however, slight changes are observed, in that the anisotropy values of both markers embedded in DPPC vesicles are decreased while those of DPH embedded in DOPC vesicles are increased (see Figs. 2 and 3). This seems to indicate slight variations in the

microviscosity of a given type of vesicles. The increase in the anisotropy values for DPH embedded in DOPC vesicles may represent an effect similar to that found in cell cultures while the decrease in the anisotropy values for DPPC vesicles may be due to the inverse isomerization effect of the molecules. They are the molecules in the most fluid parts of the system which are the first to return to the normal (fluorescent) state of the molecule, hence the apparent decrease in anisotropy.

(4) Fluorescence intensity and anisotropy of DPH embedded in mixtures of vesicles of DPPC and DOPC as function of irradiation

A good model for the simulation of the situation in cells, where the probe is situated in lipids of different nature and hence different microviscosity, we considered to be a mixture of vesicles of DPPC and of DOPC, each separately marked with DPH and later mixed together. When these mixtures were irradiated the intensity decreased and the anisotropy values increased as expected. However, neither the intensity nor the anisotropy values corresponded to the actual mixing ratio of DPPC and DOPC, even for the non-irradiated samples. Calculation of the mixing ratio for the irradiated samples showed a significant contribution of DPH in DOPC vesicles to the observed (very low) intensity, while this contribution should have been negligible as compared to the contribution of DPH in DPPC vesicles. The reason for this behaviour was found to be a transfer of DPH from one kind of vesicles to the other. This is demonstrated by the following experiment: when DPPC vesicles are labeled with DPH in the described manner an anisotropy value of 0.329 is observed; when an equal amount of DOPC vesicles without DPH is added, the intensity increases by a factor of 4 and the anisotropy decreases to about 0.23. This is only possible if a great part of the DPH molecules migrated from DPPC to DOPC. Likewise when DOPC vesicles are marked by DPH and non-labeled DPPC vesicles are added the intensity decreases to about one half of the original value while the anisotropy increases from 0.08 to 0.21. It is seen that the final anisotropy value is almost the same whether DPPC or DOPC vesicles have been marked with DPH. Different samples of vesicles gave slightly different anisotropy values for the components and hence slightly different values of the mixtures. On the basis of these results one can actually not discriminate between a transfer of the marker molecules between the two types of vesicles and a fusion of the vesicles of DPPC and DOPC with subsequent redistribution of the marker molecules within the mixed types of vesicles. Both mechanisms would yield equal results.

Irradiation of the mixtures yields rather varying results. In all cases, however, as seen in Fig. 4, there is an initial increase in the anisotropy value with irradiation, which for prolonged irradiation is followed by a (sometimes very significant) decrease in the anisotropy value, since in this case practically all the observed fluorescence is due to molecules which returned to their fluorescent state, and these are primarily the molecules in the less viscous medium.

The situation in cell cultures is quite different. In this case the exchange of DPH between different regions is much slower than between vesicles in suspension, and hence the greater increase in the polarization values with time of irradiation.

With TMA-DPH as probe, a transfer of the probe molecules takes place from DPPC to DOPC vesicles until some equilibrium distribution is reached. This is seen by the increase in fluorescence intensity of DPPC vesicles marked with TMA-

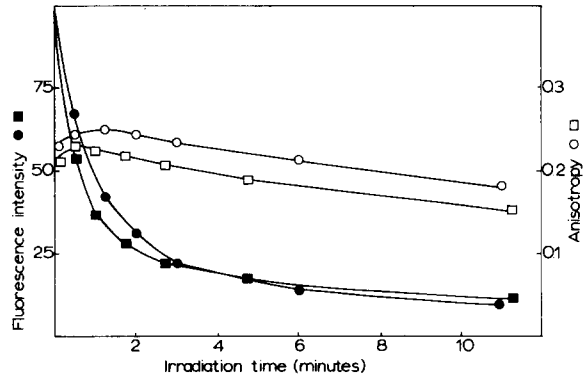

Fig. 4. Fluorescence intensity and anisotropy of DPH embedded in DPPC vesicles to which DOPC vesicles have been added (circles) and of DPH embedded in DOPC vesicles to which DPPC vesicles have been added (squares) as a function of irradiation time. Intensities of non-irradiated samples normalized to 100.

DPH upon the addition of vesicles of DOPC without probe, as well by a decrease in anisotropy. A typical example: initial value of anisotropy 0.346; final value 0.246. The anisotropy for a mixture of equal amounts of DPPC and DOPC vesicles both marked with TMA-DPH was 0.233. The reverse process, however, namely the transfer of TMA-DPH from DOPC to DPPC vesicles, seems to be excluded. Addition of DPPC vesicles to marked vesicles of DOPC changed the anisotropy only from 0.204 to 0.210. Repeated experiments yielded similar results, although, like with DPH, the absolute values changed somewhat from one vesicle preparation to the other. The results for TMA-DPH permit the decision between the two different mechanisms (transfer of marker between vesicles vs. fusion of vesicles) which could not be discriminated by the results with DPH. If fusion were a dominant mechanism the results should be the same whether TMA-DPH has been added to DPPC or to DOPC vesicles. The unidirectional change of the anisotropy (and intensity) shows that this probe can move from vesicles of a viscous medium to vesicles of a fluid medium but not vice versa.

These experiments show that the experimental location of TMA-DPH in the plasma membrane by cell disruption and separation of the fragments may be very difficult, since the marker molecules may move from the plasma membrane fragments to fragments of other parts of the cell by collisional contact and thus appear in locations at which they would not be found in the intact cell [6].

(6) Irradiation of DPH and TMA-DPH in vesicles of a mixture of DPPC and in membranes of trout intestinal cells and of rat liver mitoplasts

Fig. 5 shows the fluorescence intensity and anisotropy of DPH in suspensions of vesicles which have been prepared from a mixture of DPPC and DOPC and in suspensions of trout intestinal brush border membranes as function of irradiation time. Results obtained for suspensions of membranes of rat liver mitoplasts were very similar to those obtained for the trout intestinal brush border membranes.

It is seen that the anisotropy of DPH in these systems is only slightly affected by the irradiation,

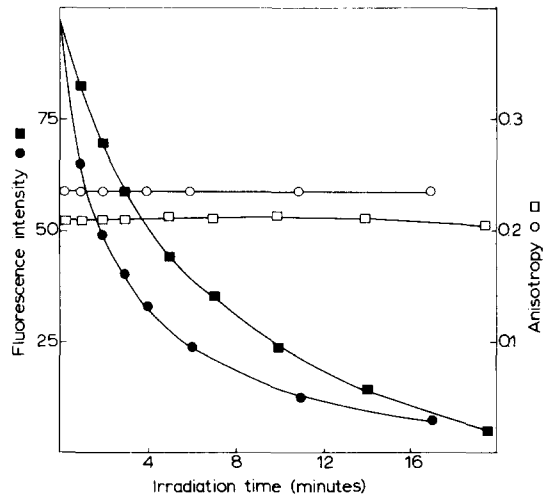

Fig. 5. Fluorescence intensity and anisotropy of DPH embedded in vesicles of a mixture of DPPC and DOPC (circles) and in trout intestinal brush border membranes (squares) as a function of irradiation time. Intensities of non-irradiated samples normalized to 100.

which indicates a rather homogeneous microenvironment of the probe molecules or else a very fast interchange of the probes within the different parts of a given vesicle or membrane.

Fig. 6 shows the fluorescence intensity and anisotropy of TMA-DPH in mitoplasts of rat liver

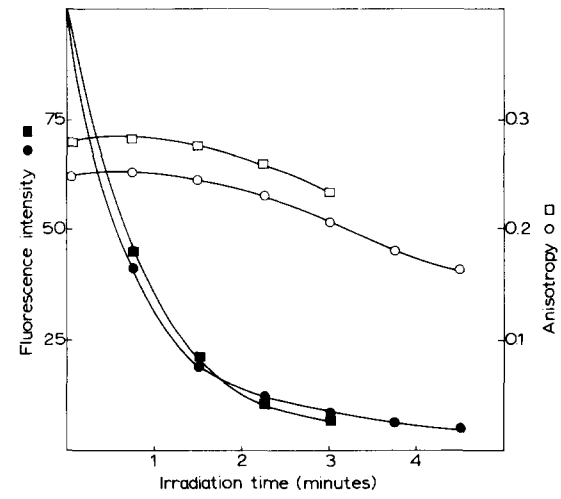

Fig. 6. Fluorescence intensity and anisotropy of TMA-DPH embedded in rat liver mitoplasts (circles) and in trout intestinal brush border membranes (squares) as a function of irradiation time. Intensities of non-irradiated samples normalized to 100.

cells as function of irradiation time. The decline is non-exponential, i.e. the rate constant of the decrease in intensity decreases with irradiation time. The anisotropy shows an initial increase followed by a significant decrease, in particular after the major part of the fluorescence intensity has been extinguished. Similar results have been obtained for TMA-DPH in membranes of trout intestinal cells. This decrease in anisotropy is similar to that observed for DPH in a mixture of vesicles of DPPC and DOPC. It may be due to the fact that a significant part or the observed fluorescence comes from the restored molecules, and hence from the more fluid parts of the membrane. It is, however, possible that the protein content of the membrane influences the results in a way which needs further elucidation. We have, however, established that the decrease is not due to radiation effects in the membrane itself. In a series of measurements we irradiated mitoplasts prior to the introduction of the marker and then started the regular measurements of intensity and anisotropy vs. time of irradiation. There was no difference between the results for the irradiated and non-irradiated membranes. Also irradiation of the membranes in the presence of the probe does not have any effect on the results. We irradiated membranes probed with TMA-DPH prior to the introduction of additional TMA-DPH molecules and then performed the intensity and anisotropy measurements; the results were practically identical with those obtained for membranes which have not been pre-treated in this manner. A remarkable decrease in the anisotropy values of TMA-DPH has been observed in many of our measurements in cell cultures. Generally they were the cells with the highest anisotropy values for which the fastest decrease in anisotropy was observed with time of measurement. The decrease in anisotropy which is at variance with the increase in anisotropy for DPH as marker may lead additional support to the assumption that the TMA-DPH stays in the plasma membrane, although the exact mechanism of the decrease requires further elucidation.

References

1 Shinitzky, M. and Barenholz, Y. (1974) J. Biol. Chem. 249, 2652–2657
2 Weinreb, A. and Travo, P. (1984) Eur. J. Cancer Clin. Oncol., in the press
3 Johnson, S.M. and Nicolau, C. (1977) Biochem. Biophys. Res. Commun. 76, 869–874
4 Van Hoeven, R.P., Van Blitterswijk, W.J. and Emmelot, P. (1979) Biochim. Biophys. Acta 551, 44–54
5 Prendergast, F.G., Haughland, R.P. and Callahan, P.J. (1981) Biochemistry 20, 7333–7338
6 Kuhry, J.G., Fonteneau, P., Duportail, G., Maechling, C. and Laustriat, G. (1983) Cell Biophys., in the press
7 Lawaczek, R., Kainosho, M. and Chan, S.I. (1976) Biochim. Biophys. Acta 443, 313–330
8 Szoka, F., Jr. and Papahadjopoulos, D. (1980) Annu. Rev. Biophys. Bioeng. 9, 467–508
9 Dickens, B.F. and Thompson, G.A., Jr. (1980) Biochemistry 19, 5029–5037
10 Schmitz, J., Preiser, H., Maestracci, D., Ghosh, B.K., Cerda, J.J. and Crane, R.K. (1973) Biochim. Biophys. Acta 323, 98–112
11 Hauser, H., Howell, K., Dawson, R.M.C. and Bowyer, D.E. (1980) Biochim. Biophys. Acta 602, 567–577
12 Lévy, M., Toury, R. and André, J. (1967) Biochim. Biophys. Acta 135, 599–613
13 Waksman, A. and Rendon, A. (1974) Biochimie 56, 907–924
14 Shinitzky, M. and Inbar, M. (1976) Biochim. Biophys. Acta 433, 133–149
15 Stubbs, C.D., Koyama, T., Kinosita, K. and Ikegami, A. (1981) Biochemistry 20, 4257–4262