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LIPID FLUIDITY AND MEMBRANE PROTEIN MONITORING USING 1,6-DIPHENYL-1,3,5-HEXATRIENE

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Summary

Experiments have been designed to challenge the use of steady-state fluorescence polarization with 1,6-diphenyl-1,3,5-hexatriene as an evaluator of the fluidity of cell plasma membranes. We used paraffinic systems, of defined structure and composition (liquid paraffin, soap bilayers and phospholipid liposomes—with and without incorporated proteins), to demonstrate that corresponding polarization values cannot be interpreted in terms of the overall fluidity of the labeled medium. In homogeneous structured paraffinic media (lipid bilayers), knowledge of the location of the probe is essential for a consistent interpretation of the observed fluorescence polarization. Due to the highly polarizable electronic structure of the diphenylhexatriene molecule, the presence of heterogeneities with potential sites for interaction (e.g., C_{18}-coated Si particles, albumin molecules, etc.) can lead to relatively high polarization values, even in isotropic media. In cellular systems, translocation experiments from labeled cells to added proteins show a rather localized peripheral distribution of the probe as well as its high affinity for hydrophobic sites of proteins. This and other arguments presented here suggest that although cellular polarization values represent an intricate average over all labeled hydrophobic regions of the cell (phospholipid bilayers, membrane proteins, etc.), these values might reflect, to a large extent, interactions of the probe with proteins from the inner periphery of the cell.

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Introduction

Although the concept of membrane fluidity still lacks an operational definition, it has recently been proposed that changes in the fluidity of the lipidic part of the cell plasma membrane could play a critical role, particularly in leukemic and transformed cells [1]. Strictly speaking, fluidity is an integral property of a medium which should be associated with both characteristic times of motions of its molecular components and with available overall free volume; whereas fluorescence polarization values have a local character (even though they represent an average of populations of the fluorophore). Nevertheless, cell membrane 'fluidity' is extensively associated with the steady-state fluorescence polarization of a labeling hydrophobic probe, 1,6-diphenyl-1,3,5-hexatriene [2,3]. Although steady-state polarization values correspond rigorously to motional randomization of the orientation of only one axis—the fluorescence polarization axis—during the lifetime of the corresponding excited electronic state, it is generally assumed that this loss of initial orientation is correlated with overall reorientations of the probe molecules, which in turn would reflect the fluidity of the medium (defined as the inverse of its viscosity). Thus, the lower the observed polarization values, the more fluid would be the labeled medium. In view of a claimed correlation between steady-state fluorescence polarization of diphenylhexatriene in cells and leukemic transformation [4], we have undertaken a critical examination of the possible significance of such cellular steady-state polarization values. However, no attempt has been made to cover that subject matter extensively. In particular, neither the possible role of cholesterol as a modulator of the fluidity of the plasma membrane lipids nor the justification for the conversion of polarization values into 'microviscosity' will be discussed here. These two topics have already been questioned thoroughly in the literature [5,6], mainly on the basis of time-dependent studies for the latter [7,8]. We have stressed rather the high anisotropy of the fluidity of lipid bilayers, as estimated from nuclear magnetic resonance experiments [9] and the importance of intermolecular interactions in condensed media, both aspects of the fluorescence polarization experiments which seem to have been underestimated in previous investigations, together with the need for detailed structural knowledge of the systems used. In other words, this paper deals with interpretation of the structural order information contained in steady-state fluorescence polarization and not on the kinetic one which can be obtained only through time-dependent analysis.

Simple steady-state fluorescence polarization experiments conducted in systems of defined structures and compositions (liquid paraffin, soap bilayers, phospholipid vesicles—with and without incorporated proteins) show that polarization values of diphenylhexatriene are highly dependent upon the structure of the medium tested and upon the presence of potential sites for hydrophobic-type interactions. In whole cells, it is thus suggested that steady-state fluorescence polarization values monitor specific interactions of the probe with some particular hydrophobic structures of the cell periphery, rather than the overall fluidity of its lipidic parts. This proposal, which designates mainly proteins (large enough to possess hydrophobic folds) as the best candidates for these interacting hydrophobic structures, is supported by the following experiments.

Addition of proteins to the labeled medium, either incorporated into liposomes or adsorbed onto the cell surface, leads to considerable increases in fluorescence polarization, strongly suggesting a high affinity of diphenylhexatriene for hydrophobic regions of proteins. We consequently hypothesized the possibility of monitoring activation of membrane-associated proteins through external stimulation. This hypothesis was tested in two cellular models: first, by monitoring activation of labeled platelet phospholipases, through changes in intracellular polarization values (Ref. 10 and Stahl, K.W., Mély, B. and Mishal, Z., unpublished data); and second, by monitoring the initial peripheral perturbation accompanying the specific triggering of normal quiescent T lymphocytes by a divalent cation ionophore, A23187 [41].

Data discussed in this paper are consistent with the proposition that membrane-associated proteins make a dominant contribution to the polarization of fluorescence of diphenylhexatriene in cells, although not excluding the lipid contributions.

Materials and Methods

1,6-Diphenyl-1,3,5-hexatriene

The molecular structure of diphenylhexatriene exhibits some important features, which are particularly relevant to the interpretation of its fluorescence polarization in labeled media. These are: (1) Its prolate ellipsoidal shape which, at least in the planar all-*trans* conformation of the electronic ground state, minimizes the inherent sterical perturbations brought about upon introduction of extrinsic probes into organized media [11]. In addition, the fact that its main molecular axis ($\vec{m}$) and the direction of the fluorescence transition moment (that we shall name hereafter the 'fluorescence axis') are nearly parallel [12] justifies the assumption that reorientations of ($\vec{m}$) affect, to the same extent, the polarization of the fluorescence. However, it must be realized that this strong departure from spherical symmetry makes polarization values observed in anisotropic structures as dependent upon the average orientation of the probe as upon the frictional constraints exerted on it by the medium. Thus, in a lamellar structure of given fluidity, widely different polarizations can be expected from the discrete orientations allowed by the anisotropic force field. (2) Its extended conjugated electronic system which, in addition to conferring on the probe its interesting spectroscopic properties, favours the occurrence of intermolecular interactions of the van der Waals type [13]. (3) The absence of any polar group and thus of permanent electronic charge, which renders this molecule practically insoluble in polar media and thus contributes to its 'polarity specific' fluorescence (e.g., almost no fluorescence in polar media and high visible fluorescence in apolar media).

Fluorescence polarization (P) measurements

Fluorescence polarization was measured with an Amino SPF-500 ratio spectrofluorometer (used in the single-beam mode), equipped with a polarization accessory and an X-Y recorder (Aminco, Silver Spring, MD). The excitation wavelength was centred at 368 nm and fluorescence polarization was computed according to the relationship:

$$P = \frac{I_{VV} - I_{VH}}{I_{VV} + I_{VH}}$$

where I_{VV} and I_{VH} represent the fluorescence intensities measured with the emission analyzer parallel (I_{VV}) or perpendicular (I_{VH}), respectively, to the polarization of the excitation beam. Fluorescence intensities were measured in arbitrary units, either from peaks of recorded spectra (at approx. 405, 427 and 455 nm) or from digital readouts (at 427 nm). No correction was made for fluctuations in intensity nor in slightly different H and V excitation intensities. The background of fluorescence intensities of unlabeled samples was always neglibible (less than or equal to 3%). To minimize reversible deactivation of the probe, all experiments were conducted in the dark, and labeled samples were never exposed to exciting radiation for periods longer than approx. 10 s. With each type of sample studied (either protein solutions or cell suspensions), dilution experiments were conducted to test for light-scattering effects. Both components of fluorescence (I_{VV} and I_{VH}) were found to be insensitive to variations in turbidity due to the restricted band pass of the emission channel. On the contrary, the relatively high intensity and polarizaton of the Rayleigh peak at 736 nm (approx. $2\nu_0$) is likely to affect in a non-linear, concentration-dependent way the polarization values obtained when recording the emission in a high band pass way (as with an Elscint MV-1 microviscometer) rather than in a narrow band pass way (as with the Aminco SPF-500 spectrofluorometer). This could explain the dependence of polarization values on the number of scattering 'particles' (either cells or albumin molecules) observed with the former apparatus (Mély-Goubert, B., unpublished data and Ref. 10).

Isotropic paraffinic medium

The paraffin used (Merck, Darmstadt, F.R.G.) was a mixture of alkanes of different chain length. Solutions of approx. 1 nM diphenylhexatriene were prepared by adding weighed amounts of crystalline diphenylhexatriene (Fluka, Buchs, Switzerland) or by adding given volumes of the fluorophore in acetone, which was removed by bubbling nitrogen into the solution overnight. No difference in polarization was detected between the two modes of diphenylhexatriene dissolution. Particles of silicon coated with C_{18}-saturated paraffinic chains (Kieselgel 60 HPLC C_{18} Revese Phase, Riedel-de Haen, Hanover, F.R.G.), approx. 10 μm in diameter (a gift from Professor K.W. Stahl, I.C.I.G., Villejuif, France), were added to the labeled paraffin solutions to obtain approximate concentrations, ranging from 0.25 to $9 \cdot 10^6$/ml. The concentrations were prepared by counting the particles with a Thoma chamber in a phase-contrast microscope.

Organized paraffinic medium

Soap lamellae. Lamellar (L_α) phase of potassium soap/water mixtures were prepared by mixing appropriate quantities of soap (10 and 18 carbon atoms in length) with distilled water, as described elsewhere [9]. Labeling was carried out by adding small amounts of crystalline diphenylhexatriene (approx. 1 diphenylhexatriene molecule to 10^3 soap molecules; these lamellar phases

being stable up to at least 1/10 impurity ratio [14]). Very thin phase sections, used for the fluorescence polarization measurements, were disposed in special optical cells of approx. 300 μl volume (from Dr. M. Delaye, Physique des Solides, Orsay, France). Under these conditions, light scattering, estimated by the spreading of a visible-light laser beam, was no greater than when cell suspensions were used.

Liposomes. Vesicular phospholipid structures were prepared by sonication (Branson W185 sonicator, at intermediate power output) at 0°C for 30 min of egg lecithin (type V-E, Sigma, St. Louis, MO) and egg lecithin/cardiolipin solutions in phosphate buffer, pH 7.4, followed by centrifugation at 100 000 × *g* for 60 min. Protein-containing vesicles were prepared similarly by adding 50 μg of lipid-free serum proteins (from Dr. H. Tapiero, I.C.I.G., Villejuif, France) to 10 mg of a 50 : 50 mixture of egg lecithin and cardiolipin. Labeling of all vesicle preparations with the probe was performed in two different ways: one by the addition of 2 μl of a 2 mM solution of diphenylhexatriene in tetrahydrofuran to the liposome preparation; the other by mixing (1 : 1) the liposome preparation with a 2 μM dispersion of the 2 mM tetrahydrofuran solution in phosphate-buffered saline, pH 7.4. No difference in polarization value was detected using the two different labeling procedures, although the first one gave higher fluorescence intensities. The stability of the liposome preparations, as evaluated by the reproducibility of the polarization values, lasted for at least 1 week when stored in the dark at 4°C.

Cellular systems

Normal human peripheral blood lymphocytes were purified, from venous blood, by Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradients [15]. CBA/H murine splenocytes and thymocytes were prepared as previously described [16]. The cell suspensions ($3 \cdot 10^6$/ml, more than 95% viable, as judged by trypan blue exclusion) were labeled by incubation with a 2 μM dispersion of diphenylhexatriene in phosphate-buffered saline for 30 min at room temperature. The labeled cells were centrifuged at 200 × *g* for 10 min and resuspended in phosphate-buffered saline at a final concentration of $4.5 \cdot 10^6$/ml.

Diphenylhexatriene-labeled murine splenocytes and thymocytes (10^8 lymphocytes/ml in phosphate-buffered saline, pH 7.4, containing 0.1% bovine serum albumin and 125 μg of deoxyribonuclease (Miles)) were digested with bovine serum pancreatic trypsin (Sigma) or with proteinase K (Merck) (final enzyme concentrations, 0.25 mg/ml) for 60 min at 37°C, and the enzymes were removed by cell washing. Temperature (37 ± 0.1°C) was controlled using a water bath.

Translocation experiments were performed in three different ways: first, a 3% solution of purified bovine serum albumin (Beringwerke, Marburg, F.R.G.) in phosphate-buffered saline was added to labeled lymphocytes, and the cells were incubated for 30 min at 37°C, then washed twice with phosphate-buffered saline. The original cell supernatant (containing the serum albumin) was retained; second, the procedure was repeated using labeled albumin and normal lymphocytes; third, the labeled lymphocytes were separated from the albumin solution by a dialysis membrane for 30 min at 37°C, and the cells

and supernatant were treated as in the first procedure. The fluorescence polarization of the cell pellets, the washed cells and the albumin supernatant was measured at 37°C. Translocation experiments with labeled and unlabeled lymphocytes and labeled and unlabeled concanavalin A or succinylated concanavalin A were performed in a similar fashion, except that cells were incubated with 50 mM α-methyl-D-mannopyranoside (Sigma) for 30—60 min at 37°C before centrifugation.

In all experiments, only reagent grade (or better) chemicals and solutions were used. Concanavalin A was supplied by Pharmacia, Uppsala, Sweden. Succinylated concanavalin A was prepared by Dr. A Kimura, University of Uppsala, Uppsala, Sweden. Different grades of purified albumin were kindly provided by Dr. J. Andersson, University of Uppsala, Uppsala, Sweden.

Results and Discussion

Fluorescence polarization of diphenylhexatriene in model paraffinic systems

The first series of model systems (paraffin, soap/water bilayers and phospholipid liposomes) permits us to sort out the effect of the degree of organization of a paraffinic medium on the steady-state fluorescence polarization of diphenylhexatriene.

Liquid paraffin represents a viscous isotropic unordered apolar medium, i.e., with no structural organization extending on time and space scales greater than the molecular ones. The lipidic region of soap/water bilayers, on the other hand, represents an organized paraffinic medium with a long range one-dimensional order. In these lamellae, the structure and dynamics of which are thoroughly characterized down to the molecular level [17], packing and motions of constituent molecules are highly anisotropic and result in the existence of a fluidity gradient which increases from the paraffin/water interface. As molecular packing and motions in phospholipid liposomes appear similar at the molecular level, their finite radius of curvature permit us to test the sensitivity of diphenylhexatriene fluorescence for the intermediate range order of the paraffinic chains in the vesicular structure.

The data in Table I show that, among these three paraffinic media, almost no polarization values are associated with the anisotropic lamellar structures of the two soap/water L_α phases (C_{10} and C_{18}), and not with the isotropic liquid; values associated with phospholipid liposomes being similar to the liquid paraffin ones. Actually, these results can be rationalized, by considering both the high anisotropy of the bilayer and the complete hydrophobicity of the diphenylhexatriene molecule, which makes its exposure to the water medium energetically unfavorable [18]. Thus, we propose that the most probable disposition of diphenylhexatriene in lipid bilayers is in the centre of the structure which is both its most hydrophobic and most fluid part. Although at variance with a previously assumed orientation of the probe parallel to the amphipatic molecules [2,12]—orientation which would expose the probe to the water medium known to penetrate down towards the first carbon atoms [19]—our proposal is consistent with the following observations. (1) No chain-length effect on polarization values was observed when going from the C_{18} to the C_{10} bilayer, in spite of the fact that the width of the paraffinic

TABLE I

FLUORESCENCE POLARIZATION OF DIPHENYLHEXATRIENE IN PARAFFINIC MODEL SYSTEMS

Fluorescence polarization values are given as the mean ± S.E. (three to six determinations).

Paraffinic system	Fluorescence polarization value (37 ± 0.1°C)
Liquid paraffin	0.075 ± 0.005
Lamellar L_α phase of soap/water mixture	
C_{10}	0.010 ± 0.005 *
C_{18}	0.020 ± 0.005 *
Egg lecithin liposomes	0.083 ± 0.010
Liquid paraffin containing C_{18}-coated Si particles **	0.170 ± 0.005
Liposomes with incorporated serum proteins	0.116 ± 0.010

* Measurements were made at 50°C, which is the lowest temperature of the C_{18}-L_α phase.
** The number of C_{18}-coated Si particles was adjusted to 0.25—9 · 10^6 particles/ml.

medium in the latter case is small enough—as compared to the length of the diphenylhexatriene molecule (e.g., approx. 19 Å compared to 14 Å)—to expose it to the water. (2) Early crystallographic work [20] has shown that completely apolar molecules dissolve in the hydrophobic centre of the lamellae; whereas incorporation in an orientation parallel to the hydrocarbon chains was observed only with molecules having one polar group to anchor them at the paraffin/water interface. (3) Assuming a parallel orientation of diphenylhexatriene in phospholipid bilayers, only on the basis of molecular shape, has led some authors [21] to select a very unnatural angle of wobbling diffusion (e.g., 70° with respect to the normal) in order to interpret their data consistent with almost isotropic orientations of the probe (in agreement with our proposed location of diphenylhexatriene in the most disordered fluid part of the lamella). (4) Steady-state [22] as well as time-resolved experiments [7], and even a recent theoretical analysis of fluorescence anisotropy experiments [23], agree on almost free anisotropic reorientations of the fluorescence axis of diphenylhexatriene in lipid bilayers, although corresponding interpretations range from 'an isotropic hydrocarbon medium' to the 'possibility for steady-state fluorescence anisotropy data, to furnish information on the structural order in membranes' (in the latter work, order parameters estimated from steady-state fluorescence polarizations of the probe agree quite well with those obtained, from deuterium magnetic resonance studies [24], at the methyl end of the hydrocarbon chains). Thus, considering that the most probable partition of diphenylhexatriene in lipid bilayers is in the hydrophobic centre of the structure, we interpret the observed increase in polarization when going from a planar to a curved paraffin/water interface as reflecting the sterical constraints originating from curvature effects which are actually expected to be maximum along a direction perpendicular to the radius of curvature *.

* This is further supported by the observed significant difference in polarization values between two types of unilamellar labeled liposomes of different diameters (approx. 250 Å and 1000 Å; Mély-Goubert, B., Freedman, M.H., Kämpe, O. and Peterson, P.A., unpublished results).

TABLE II

FLUORESCENCE POLARIZATION OF DIPHENYLHEXATRIENE IN HETEROGENEOUS POLAR/APOLAR SYSTEMS

The proteins were labeled at a concentration of 0.1—3%. The cells were labeled at a concentration of 4—5 · 10^6/ml. The polarization values represent the mean ± S.E. (6 to 30 determinations).

System (diphenylhexatriene-labeled)	Fluorescence polarization value (37± 0.1°C)
Bovine serum albumin *	0.367 ± 0.009
Concanavalin A	0.380 ± 0.001
Lymphocytes **	0.202 ± 0.008

* An additional 18 purified diphenylhexatriene-labeled proteins (M_r = 25 000—250 000) gave fluorescence polarization values ranging from 0.280 to 0.400 (0.339 ± 0.074), at 37 ± 0.1°C.

** Lymphocytes from human peripheral blood, CBA/H murine spleens and thymuses.

The last paraffinic system tested (liquid paraffin plus C_{18}-coated silicon particles) emphasizes the additional importance of compositional heterogeneities of the medium in the observed polarization values. Thus, in a lableled isotropic liquid of low fluorescence polarization (approx. 0.075), addition of inert particles led to more than a 100% increase in polarization value (approx. 0.170, Table I), irrespective of the number of particles. Taking advantage of the simplicity of the system used (both structurally and chemically), which makes improbable important undetectable changes in its structure or its composition upon addition of the particles, one can associate this increase in polarization to orientational restrictions of the fluorescence axis of the probe introduced by intermolecular interactions with the C_{18} chains. Such oriented interactions can actually be strong enough to lead to stable observable associations in solution, as already shown by ligh-scattering studies of mixtures of alkanes and aromatic molecules [25].

This property of the diphenylhexatriene molecule to make strong van der Waals type interactions was investigated further with aqueous protein solutions (e.g., bovine serum albumin and concanavalin A). The upper part of Table II shows that these water-soluble proteins can be efficiently labeled by adding varying amounts of them to a (non-fluorescent) buffer dispersion of diphenylhexatriene, leading to high intensities of highly polarized fluorescence of the probe over the very low intrinsic fluorescent signal of the unlabeled proteins. Moreover, denaturation of the labeled albumin (by either 0—8 M urea or by 0—6 M guanidine hydrochloride) led to corresponding decreases in both the intensity and the polarization of the fluorescence signal, down to their background level (results not shown). These results taken together strongly support the view that in its native conformation, apolar folds of the albumin molecule constitute sufficiently hydrophobic regions to allow fluorescence of diphenylhexatriene, and that these hydrophobic 'pockets' are progressively exposed to the water medium upon denaturation *.

* That the observed fluorescence signal refers mainly to a labeling of hydrophobic crevices of proteins and not to a superficial dissolution of the probe into 'contaminating lipids', is strengthened by the following (in addition to the fact that a rather high concentration of such contaminating lipids would be necessary to exclude a protein contribution): (1) Similar fluorescence signals, both in intensity and in polarization (within 10%) were observed with albumins of different origins, up to a highly purified one practically devoid of lipids, although small differences could be attributed to the different purification procedures. (2) Among a wide range of proteins and glycoproteins examined, the latter devoid of contaminating

Fluorescence polarization of diphenylhexatriene in cellular systems

In spite of the considerable number of cellular systems investigated with this technique (for a review, see Ref. 26), the basic question of the topographical specificity of cellular fluorescence polarization values of diphenylhexatriene still appears controversial. Thus, the possibility of attributing the measured polarizations to a particular cell region is not only questioned by previous experiments with tritiated diphenylhexatriene [27], but also by the hydrophobic nature and the unbulky molecular shape of this fluorophore, which make highly probable its distribution through most apolar regions of the cell.

We first looked for possible trivial contributions to the cellular fluorescence signal from probe populations localized on the external part of the cell surface (e.g., in the hydrophobic crevices of water-soluble proteins and/or glycoproteins of the outer cell surface). Murine lymphocytes were thus incubated with either trypsin or proteinase K before and after labeling of the cells. Trypsin cleaves critical lysine and arginine residues of proteins at the cell membrane surface; whereas, proteinase K has a much wider specificity for the cleavage of cell surface proteins. Enzymatic cleavage of the surface of the lymphocytes did not result in significant changes either in fluorescence polarization or in the intensity of the probe's fluorescent signal, compared to untreated labeled cells. This demonstrates that the major proteins and glycoproteins on the cell surface do not interfere significantly either in the measured polarization or in the incorporation of the probe populations contributing to the recorded signal.

In a second step, the question of the probe internalization was approached through its inversely related consequence, namely through an evaluation of the probe's accessibility toward externally added proteins. First, labeled cells (polarization value = 0.196) were incubated with an aqueous solution of serum albumin, known to adsorb to cell surfaces and to act as a carrier for poorly water-soluble substances [28]. After washing the cells to remove the adsorbed proteins, all the probe's fluorescence signal was found in the supernatant (Table III A) but with a higher polarization value (0.368) than the cellular one, actually very close to the value obtained for labeled serum albumin (Table II). No diphenylhexatriene fluorescence could be detected in the previously labeled cellular fraction over the intrinsic fluorescent background. This finding is actually in line with previous studies which showed translocation of the probe between two different cell types [29] and between a virus and lymphoid cells [30]. Further investigations, made possible by the greatly different polarization values associated with cells (0.202 ± 0.008) and with the albumin molecules (0.367 ± 0.009) (Table II), showed such translocation to occur both ways, i.e., from labeled serum albumin to unlabeled cells (although to a lesser extent, Table III B), and from labeled cells to unlabeled serum albumin (Table III A). It was found that close interactions

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lipids (Kimura, A., private communication) (Table II), only the ones which obviously lacked hydrophobic folds (i.e., mostly the smaller ones) did not lead to a corresponding labeling with diphenylhexatriene.

TABLE III

TRANSLOCATION OF DIPHENYLHEXATRIENE

The fluorescence polarization values represent the mean ± S.D. of at least triplicate measurements.

System	Fluorescence polarization value (37 ± 0.1°C)
(A) Diphenylhexatriene-labeled murine splenocytes + serum albumin [a]	0.196 ± 0.004
washed splenocytes	0.000
supernatant	0.368 ± 0.006
(B) Diphenylhexatriene-labeled serum albumin + murine splenocytes [b]	0.365 ± 0.004
washed splenocytes	0.194 ± 0.003
supernatant	0.380 ± 0.005
(C) Diphenylhexatriene-labeled serum albumin (dialysis tubing) [c]	
murine splenocytes [c]	0.366 ± 0.006
washed splenocytes	0.000
supernatant	0.365 ± 0.007
(D) Diphenylhexatriene-labeled murine splenocytes + concanavalin A [d]	
α-methyl-D-mannopyranoside [d]	0.205 ± 0.004
washed splenocytes	0.210 ± 0.005
supernatant	0.390 ± 0.007

[a] A 3% solution of serum albumin in phosphate-buffered saline was added to labeled CBA/H murine splenocytes ($4.5 \cdot 10^6$/ml) for 30 min at 37°C. The cells were washed three times with phosphate-buffered saline. The supernatant was from the first centrifugation.

[b] A 3% solution of labeled serum albumin was added to CBA/H murine splenocytes ($4.5 \cdot 10^6$/ml) for 30 min at 37°C. The cells and supernatant were treated as in a.

[c] The conditions were as in a, except that the labeled serum albumin was placed in a dialysis sac.

[d] Concanavalin A (150 μg/ml) was added to labeled CBA/H murine splenocytes for 5 min at 37°C. Then 50 mM α-methyl-D-mannopyranoside was added to the cells for 30—60 min at 37°C. The cells and supernatant were treated as in a.

between the cell surface and the 'carrier molecule' were necessary for translocation of the probe to occur. Thus, keeping labeled cells apart from the albumin by a dialysis membrane prevented translocation of the probe (Table III C). The distance-dependence of this phenomenon was subsequently stressed with concanavalin A (a T lymphocyte mitogenic lectin), which is considered to interact in close contact with the cell surface, only at relatively high agglutinating concentrations. Effective labeling of concanavalin A molecules (eluted with α-methyl-D-mannopyranoside), demonstrating translocation of the probe from initially labeled cells, was significant only when high doses (greater than 50 μg/ml) of the lectin were used (Table III D). Interestingly enough, these experiments support the previous suggestion that binding of concanavalin A would involve hydrophobic interactions with the surface concerned [31]. It was strengthened further by the absence of any detectable translocation when succinylated concanavalin A (even at very high doses, greater than 200 μg/ml) was used instead, confirming the view that succinylation might prevent the protein from interacting closely with the cell surface.

It is thus highly surprising that no translocation was reported upon the interaction of diphenylhexatriene labeled lymphocytes with serum proteins [29]. In particular, the fact that protein polarization values are considerably higher than any cellular polarization values (Table II) observed up to now casts some doubts on the possibility of monitoring rapid increases in 'mem-

brane fluidity' (lowering of polarization value) upon interaction of labeled lymphocytes with several lectins [32]. The average polarization value for freshly prepared normal lymphocytes (0.202 ± 0.008, Table II) did not change significantly when normal human lymphocytes were maintained in tissue culture (polarization value = 0.204 ± 0.005). When normal human lymphocytes were cultured, in the presence of a mitogenic dose of concanavalin A for 48—72 h, a slight decrease (approx. 8%) in the polarization value was observed. This observation supports the view that lectin triggering does not induce early [41] and late changes in membrane bound proteins, to which we consider that the probe should be most sensitive. In this connection, it is interesting to point to a recent biochemical analysis of the plasma membrane of lectin-activated B and T lymphocytes [33] which showed that the main changes in membrane patterns upon lectin stimulation were in the ganglioside and glycolipid composition and not in the protein one.

Besides showing the unexpected importance of translocation processes, our results show that at least the fraction of diphenylhexatriene molecules which contributes to the fluorescent signal stays readily accessible to an albumin extraction. Even if there is an appreciable internalization of the probe in intracellular hydrophobic regions, it seems that the observed fluorescence signal could be attributed to a rather localized peripheral population of the probe. Considering the high affinity of diphenylhexatriene for apolar regions of proteins together with the uniquely high concentration of this class of molecules that the cell plasma membrane realizes [34] strongly suggests the possibility of monitoring structural changes of such labeled peripheral proteins induced upon cell plasma membrane activation. This was tested in two different cellular systems, platelets and lymphocytes. With labeled platelets, both non-specific (with octanol) and specific (using a small, non-translocating polypeptide, bradykinin) activation of membrane-linked phospholipases were monitored through transient reversible fluorescence polarization changes (Ref. 10; and Stahl, K.W., Mély, B. and Mishal, Z., unpublished data). With labeled lymphocytes, transient reversible changes in fluorescence polarization were observed upon addition of a low, non-toxic, mitogenic dose of a divalent cation ionophore (A23187) [41]. The fact that phospholipid liposome exhibited transient increases only in fluorescence intensity (and not in polarization value) upon penetration by the ionophore [41], agrees with an involvement of membrane-associated proteins in the detected cellular changes *. This view which suggests that the initiation of cell activation by the divalent cation ionophore (A23187) could involve such proteins, favors an interpretation of the ionophore specificity towards different lymphocyte subpopulations in terms of a lack of Ca^{2+}-dependent, membrane-associated proteins (in the non-responding populations), rather than in terms of highly different solubility properties of the ionophore in their plasma membrane lipids (to our present knowledge, available data on

* Recently, Klausner et al. [35], using vesicles prepared from lipid extracts of cell membranes and reconstituted with $(Na^+ + K^+)$-ATPase, also showed how critical the protein is to the fluorescence polarization of diphenylhexatriene, although no report was made of time-dependent changes induced upon activation.

the phospholipid composition of the cell plasma membrane of different lymphocytes do not point to strong differences in phospholipid composition).

Conclusion

Steady-state fluorescence polarization of diphenylhexatriene does not correlate with the fluidity of the labeled medium [36]. Even in relatively simple paraffinic media, important differences in polarization can originate from factors not directly related to the viscosity of the medium. Apart from differences which could arise from variations in the fluorescent excited-state lifetime, which steady-state methods can hardly reach, our experiments stress the importance of two previously non-analyzed parameters which appear to control the steady-state fluorescence polarization of diphenylhexatriene. (1) The structural organization of the labeled medium which will determine the partition of the probe, making its fluorescence polarization as much 'structure-dependent' as 'fluidity-dependent'. (2) The existence of potential sites for specific interactions in which the diphenylhexatriene molecule can be involved due to its extended conjugated electronic structure. In that case, such hydrophobic and/or van der Waals type interactions can be strong enough to lead to the highest polarizations observed, in spite of the isotropy and the fluidity of the system which allows reorientations around the three cartesian axes.

These findings, however, furnish a rationale to interpret fluorescence polarization experiments conducted with diphenylhexatriene in cells. Considering that the fluorophore reports mainly from the cell periphery (i.e., hydrophobic regions of the plasma membrane) as supported by the translocation experiments, and that most of the corresponding lipids exist in a bilayer structure (as supported both by theoretical [37] and experimental data (reviewed in Ref. 38) with which are associated very low polarization values (between 0.0 and 0.085), then the relatively high polarization values associated with normal cells (approx. 0.202) witness a contribution of 'high polarization structures' to the overall signal. Because of the high affinity of the probe for proteins large enough to possess hydrophobic crevices (to which are associated the highest observed polarization values, i.e., between 0.360 and 0.400 at 37°C) and of the considerably high concentration of proteins found on the inner side of the plasma membrane, we propose that the signal from diphenylhexatriene in cells contains an important contribution from protein and/or lipoprotein regions.

In spite of the considerable number of different proteins found at the level of the cell plasma membrane (integral, transmembrane, submembranous), this proposal, which does not exclude the lipid contribution * to the observed polarization values, designates highly folded proteins (or enzymes) as a critical class. It is thus particularly relevant to notice that in the two cellular systems reported (platelets and lymphocytes), the observed time-dependent changes in polarization are more consistently interpreted in terms of structural changes

* Recent morphological studies on the effects of peripheral lipid extraction by detergent suggest, however, that their importance—at least in the maintenance of the cell shape—may have been overestimated over the protein one [39].

induced in membrane-associated proteins. In that view, we proposed recently [40] that the smaller average polarization values of diphenylhexatriene in leukemic cells reveal an important alternation in this class of proteins.

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