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Membrane Aging during Cell Growth Ascertained by Laurdan Generalized Polarization

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The sensitivity of the fluorescent probe Laurdan to the phase state of lipids has been utilized to detect modifications in the composition and physical state of cell membranes during cell growth. In phospholipid vesicles, the Laurdan emission spectrum shows a 50-nm red shift by passing from the gel to the liquid–crystalline phase. The Generalized Polarization (GP) value has been used for the data treatment instead of the ratiometric method common in investigations utilizing other fluorescent probes that display spectral sensitivity to medium properties. The GP value can be measured easily and quickly and possesses all the properties of "classical" polarization, including the additivity rule. Once Laurdan limiting GP values have been established for the gel and the liquid–crystalline phase of lipids, the quantitative determination of coexisting phases in natural samples is possible. In the present work the observation of a relevant decrease in the fractional intensity of the liquid–crystalline phase in K562 cell membranes during 5 days of asynchronous growth is reported. A decrease in the "fluidity" of cell membranes in K562 cells kept in culture for several months is also reported. The procedure developed for labeling cell membranes with Laurdan is reported and the influence of cell metabolism on fluorescence parameters is discussed. Also discussed is the influence of cholesterol on Laurdan GP. © 1992 Academic Press, Inc.

INTRODUCTION

The phospholipid matrix constitutes the environment for cell membrane functions, wherein membrane proteins display their activity. Many studies of biochemical and medical interest are concerned with the assessment of membrane dynamics, in particular, the physical state of the lipids and, although there is a lack of a precise physical definition, the "fluidity" variations accompanying physiological and pathological events [1–4].

Membrane functions are known to be influenced by the phase state of phospholipids, i.e., by their dynamic aspects. Modifications of membrane fluidity can control the expression of proteins and receptors exposed on the cell surface [5, 6]. For phospholipids in the bilayer aggregation form, two phase states have been described, the gel and the liquid–crystalline state. In natural membranes, the high variability of phospholipids—different acyl residues and polar heads—gives rise to microheterogeneity in their phase state. The problem arises whether the two phases are mixed and their properties averaged or whether a segregation between domains exists in the membrane plane, each domain with its specific properties. The partitioning of proteins into different domains can modulate their functional activities and lateral diffusion.

Fluorescence spectroscopy offers several advantages for the study of membrane dynamics. Among these advantages are the high sensitivity of the technique and the virtual absence of perturbation of membrane structure due to the probes themselves. Also important are the responsiveness of fluorescence parameters to the physical properties of the environment and the possibility of resolving spectroscopic parameters arising from sample heterogeneity.

While in model systems several probes have well-defined properties in each individual phase, in biological samples the same probes fail in the observation and quantitation of coexisting separate lipid domains of different phase states. The widely used probes 1,6-diphenyl-1,3,5-hexatriene (DPH)[2] [7–10] and parinaric acids [11] are good examples of this deficiency. These probes are commonly used to obtain information on averaged fluidity properties of natural membranes by means of fluorescence polarization measurements. Because of the complex photophysical properties of these probes [11–13], the resolution and quantitation of coex-

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[2] Abbreviations used: DPH, 1,6-diphenyl-1,3,5-hexatriene; Laurdan, 6-dodecanoyl-2-dimethylaminonaphthalene; DMPC, dimyristoylphosphatidylcholine; PBS, phosphate-buffered saline solution; Me₂SO, dimethyl sulfoxide.

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isting lipid phases, while possible in principle, require long and delicate measurements, which are not appropriate for investigations of biological samples. Nevertheless, results obtained by the continuous lifetime distribution of DPH have been used to estimate membrane microheterogeneity as a function of various physiological or pathological events. Modifications of cell membrane heterogeneity have been reported during the early stage of erythroid differentiation [14] in stimulated human granulocytes [15], and in erythrocyte ghosts, the degree of heterogeneity has been found to be related to cholesterol concentration [16]. The width of the DPH lifetime distribution has been used to detect oxidative damage induced on erythrocyte ghosts by low doses of ionizing radiation [17].

Recently, a new probe has been synthesized [18] and characterized [19–22] for studies of membrane structure and dynamics, the 6-dodecanoyl-2-dimethylamino-naphthalene (Laurdan). The advantage of using Laurdan is mainly due to its spectral sensitivity to the properties of its environment. Laurdan is an amphiphilic molecule, with the lauric acid tail anchored into the membrane, and with the polar fluorescent moiety at the level of the phospholipid glycerol backbones. Laurdan possesses a high quantum yield in membranes and in organic solvents, but a negligible fluorescence in aqueous environments. Laurdan steady-state excitation and emission spectra display characteristic features related to the phase state and to the dynamics of its environment [19–22]. In mixed-phase phospholipid vesicles Laurdan steady-state excitation and emission spectra can be quantitatively resolved into two spectral components that correspond to the gel phase and to the liquid–crystalline phase. The separation of Laurdan spectral components and the possibility of selectively exciting probe molecules in different environments has been used to develop the concept of Generalized Polarization (GP) [21, 22], expressed as $GP = (I_g - I_l)/(I_g + I_l)$, where I_g and I_l are the fluorescence intensities measured at the maximum emission characteristic of the probe in gel and liquid–crystalline phospholipids and typically at 440 and 490 nm, respectively. Analogous to the fluorescence polarization, after selectively exciting Laurdan molecules surrounded by lipids in the gel or in the liquid–crystalline phase, the GP value will reveal if the initial photoselection has been maintained or lost [21]. In this second case, different GP values obtained using different excitation wavelengths can indicate the coexistence of different environments. With the GP method we measure steady-state fluorescence intensity at different wavelengths, as compared to classical polarization measurements wherein the intensity values are obtained using different directions of excitation and emission polarizers. A major advantage of GP is the possibility of using all the steady-state and dynamic information contained in the fluorescence polarization, avoiding the use of polarizers, which appreciably reduce

excitation and emission intensities, and with no need of correction for the scattering contribution, generally relevant when using biological materials. The information contained in the GP value cannot be obtained by the ratiometric method used for other fluorescent probes that display spectral sensitivity, for instance, to calcium concentration or to pH values. By measuring the GP value the ad hoc calibration curve needed in the ratiometric method can also be avoided. Still, a single GP point is obtainable in a few seconds, another relevant advantage especially when compared to DPH lifetime measurements, which take hours to perform and are technically complex. Moreover, because Laurdan spectral properties are neither affected by pH values from 4 to 10 nor by the type of phospholipid polar residues and acyl chains [22], characteristic GP values for the gel and for the liquid–crystalline phase can be determined and used for the quantitative resolution of coexisting phases.

Here we present a study of cell membrane phase states using Laurdan GP at different times of cell growth after the medium renewal. We also compare cell membrane phase states after different periods of subculture during cell line "aging."

The method and the kinetics of cell labeling are discussed, together with the problem due to modification of Laurdan fluorescence response in natural membranes where cholesterol is present.

METHODS

The K562 subclone S [23] was maintained at 37°C in RPMI 1640 medium, supplemented with 10% fetal calf serum, 5 µg/ml penicillin, 5 µg/ml streptomycin, 2 mM glutamine, and routinely subcultured every 3 days.

Cell labeling with Laurdan. Harvested cells were washed three times with PBS (phosphate-buffered saline solution, Flow Laboratories, UK). If the cells were to be fixed, the cell pellet was resuspended in 1 ml of PBS containing 4% paraformaldehyde, and after 5 min at room temperature cells were washed twice with an excess volume of PBS. The labeling buffer was freshly prepared by adding 10 µl of a 2 mM stock solution of Laurdan (Molecular Probes, Inc., Eugene, OR) in dimethyl sulfoxide (Me₂SO; Sigma, St. Louis, MO) to 50 ml of PBS. The Laurdan stock solution was renewed every 3–4 weeks. The cell pellet (4 × 10⁶ cells), fixed or simply washed, was resuspended in 3 ml of the labeling buffer. Final concentrations of Me₂SO and Laurdan in PBS were 0.002% (vol%) and 0.4 µM, respectively. After 30 min of incubation in the dark, cells were centrifuged, resuspended in 3 ml of PBS, and equilibrated for 10 min at the temperature of measurement in the fluorometer. For each sample a blank was prepared with the same number of cells in Laurdan-free PBS.

Laurdan-labeled liposomes. Multilamellar phospholipid vesicles were prepared by mixing the appropriate amounts of chloroform solution of DMPC (dimyristoylphosphatidylcholine, Avanti Polar Lipids, Inc., Pelham, AL), cholesterol (Sigma), and Laurdan and then evaporating the solvent by nitrogen flow. The dried samples were resuspended in PBS, heated to 70°C, and vortexed. All samples were prepared in red light and used immediately after preparation. The final lipids and probe concentrations were 0.3 mM and 0.3 µM, respectively. Chloroform was spectroscopic grade.

Fluorescence measurements. Laurdan excitation and emission spectra in K562 cells were obtained using a GREG 200 fluorometer

and the accompanying software (ISS, Inc., Champaign, IL). Mono-chromator bandpasses were 8 nm. For each sample the corresponding blank spectra were acquired and subtracted from the fluorescence spectra. After the correction factors for the excitation and emission spectra were found to be negligible, the spectra were no longer corrected for the monochromator response, but only for the lamp intensity variations. The Generalized Polarization values (GP) were calculated as

$$GP = (I_{435} - I_{490})/(I_{435} + I_{490}), \qquad (1)$$

where I_{435} and I_{490} are the emission intensities at 435 and 490 nm, respectively; excitation was 340 nm. During temperature equilibration and measurements, samples were continuously stirred. Temperature was controlled to $\pm0.1°C$ by a water circulating bath. GP values were acquired using ISS, Inc., software, integrating the readings to a SD ≤ 0.002.

RESULTS

The procedure used to label cells with Laurdan was established following several preliminary observations. First, the procedure used to label cells with DPH [14] could not be used with Laurdan. The DPH labeling protocol involves the addition of a small aliquot of a chloroform solution of the probe in the buffer followed by removal of the solvent from the buffer by nitrogen bubbling. This procedure did not work with Laurdan, either because of a possible affinity of the probe for the glass tube or because of a steric hindrance to the diffusion of the probe into the cell membranes. Second, Me_2SO was used instead of chloroform or ethanol because of the absence of effects on cell viability after Me_2SO treatment [24]. Moreover, after dilution experiments we established that the Me_2SO concentration in the PBS labeling buffer (0.002%) did not affect the Laurdan emission parameters in cell membranes.

Since Laurdan has a very low quantum yield in aqueous environments, the time course of the labeling can be followed as the increase of fluorescence intensity as a function of time. In Fig. 1A Laurdan emission spectra in K562 cells at 20°C are reported at different times after cells have been resuspended in the labeling buffer. The blank intensity is also reported. In Figure 1B the time courses of the average emission intensity and of the GP value are plotted as a function of time. The Laurdan emission shape and the emission maximum change during the incubation time. Both the fluorescence intensity and the GP value increase with time. In Fig. 2A the time evolution of Laurdan emission in K562 cells, centrifuged and resuspended in Laurdan-free PBS after 30 min of incubation in the labeling buffer, is reported. In Figure 2B the corresponding average total emission intensity and the GP value are reported as a function of time after the removal of the labeling buffer. A fluorescence intensity decrease can be observed while the GP value is constant. The time course of the labeling was also measured in K562 cells fixed with paraformaldehyde (Fig. 3). In fixed unwashed cells the average to-
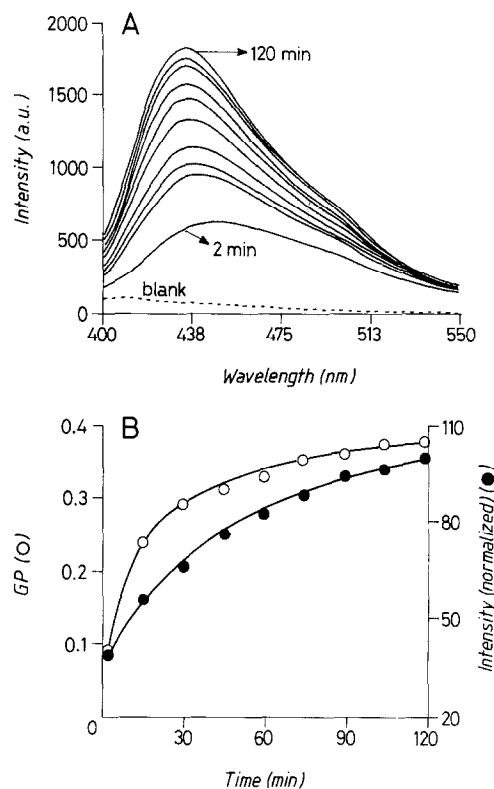

FIG. 1. Time course of K562 cells labeled with Laurdan at 20°C. (A) Emission spectra of Laurdan in K562 cells at different times after resuspension in the labeling buffer (continuous line) without blank subtraction. Also reported is the emission spectrum of unlabeled K562 cells (dotted line). (B) Average fluorescence intensity (●) and GP values (○) of Laurdan-labeled K562 cells as a function of time after resuspension in the labeling buffer. The average emission and the GP values were calculated after blank subtraction. Excitation at 360 ± 16 nm.

tal fluorescence increases with time, while in fixed cells washed after 30 min of incubation in the labeling buffer the average total emission shows a small decrease. Under both conditions the GP value is practically constant. The small increase in GP value observed in Figs. 2 and 3 during the first 10 min can be attributed to temperature equilibration.

After these labeling experiments, all measurements were performed with cells incubated for 30 min in the labeling buffer, then centrifuged, resuspended in Laurdan-free PBS, and equilibrated at the temperature of the experiment in the fluorometer for 10 min.

Natural fluorescence present in cells, with excitation and emission spectra in the same wavelength ranges of Laurdan spectra, suggests the necessity of blank subtraction. The blank subtraction affects the Laurdan GP value only when the contribution of intrinsic fluorescence to the total emission is relevant, i.e., both in the case of very fast cells labeling, if the "labeling buffer" is not removed, and in the case of living cells measured a long time after the labeling buffer removal. Nevertheless, since the present instrumentation allows rapid and
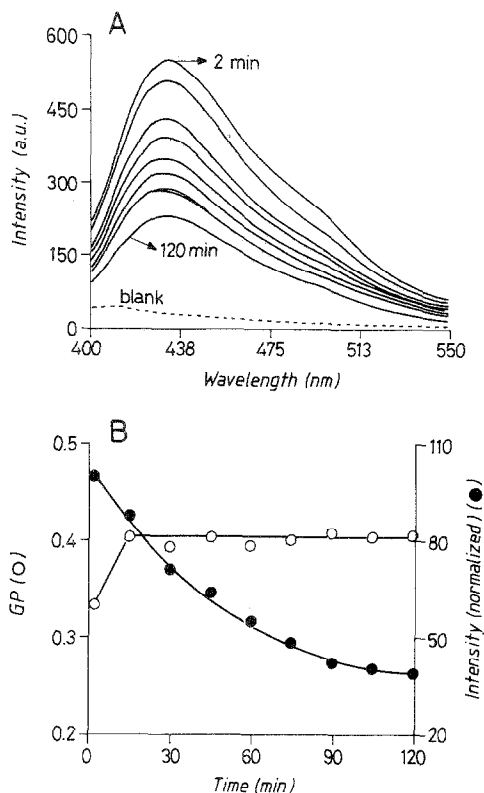
FIG. 2. (A) Laurdan emission in K562 cells as a function of time after the removal of the labeling buffer (continuous line). Cells were incubated for 30 min in the labeling buffer and then washed and resuspended in Laurdan-free PBS. Emission from unlabeled K562 cells is also reported (dotted line). Starting times of spectra acquisition were 2, 15, 30, 45, 60, 75, 90, 105, and 120 min. (B) Average fluorescence intensity (●) and GP values (O) calculated from the above spectra after blank subtraction. Excitation at 360 ± 16 nm.

facile blank subtraction and the number of cells required is small, in the present work we always subtracted the blank contribution to the total emission.
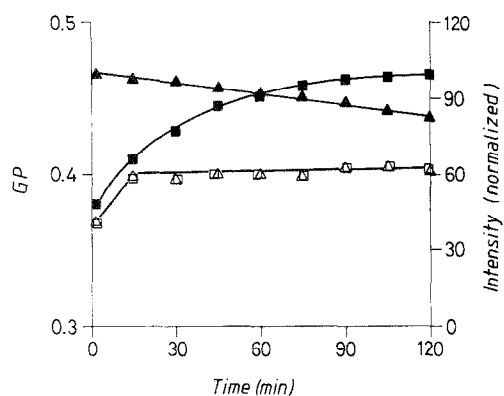

FIG. 3. Average Laurdan emission intensity (■, ▲) and GP value (□, △) in K562 cells fixed with paraformaldehyde as a function of time and after blank subtraction. Cells were measured in the labeling buffer (■, □) or washed and resuspended in PBS after 30 min of incubation in the labeling buffer (▲, △). Measurements were performed on cells 96 h after the medium renewal.
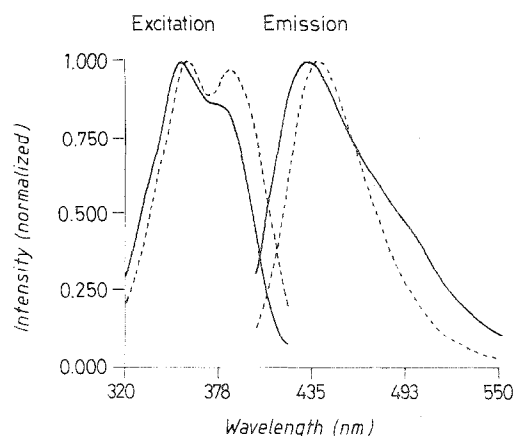

FIG. 4. Normalized Laurdan excitation and emission spectra in K562 cells at 20°C and after blank subtraction (continuous line) and in DMPC vesicles at 5°C (dotted line). K562 cells were labeled using the procedure reported under Methods. K562 cells were utilized after 48 h from the growth medium renewal.

Laurdan excitation and emission spectra in K562 cells at 20°C and after blank subtraction are shown in Fig. 4. The spectra display some peculiar features when compared to spectra obtained with Laurdan in phospholipid vesicles. A 5-nm blueshift of the Laurdan emission spectrum can be observed. The emission maximum in K562 cells was at 435 nm, while the emission maximum observed in gel phase phospholipid vesicles was at 440 nm [19–22]. From the shape of the emission spectrum and from the GP values (Figs. 1–3) we can also observe that cell membranes appear to be more similar to phospholipid vesicles in the gel phase [19–22], while we expect the living cell membranes to be fluid, close to the liquid–crystalline phase of vesicles.

In a previous work [22], characteristic Laurdan GP values in phospholipid vesicles have been determined to be about $GP_g = 0.6$ and $GP_l = -0.2$, for the gel and for the liquid–crystalline phase, respectively, and independent of the phospholipid composition and the pH value. These GP values can be used to quantify the two phases in cell membranes,

$$GP = xGP_g + (1 - x)GP_l, \qquad (2)$$

where x is the fractional intensity of the gel phase [22]. If we use these GP_g and GP_l values to quantify the two phases in cell membranes we obtain a fractional intensity of liquid–crystalline phase of 35% for cells at 20°C after 48 h from the medium renewal.

With the aim of investigating the origin of the relatively high Laurdan GP value and of the 5-nm blue shift of its emission maximum, we acquired Laurdan spectra in phospholipid vesicles composed of DMPC alone or with 30 mol% cholesterol, which is close to the cholesterol concentration in erythrocyte membranes. In vesicles composed of DMPC with 30 mol% cholesterol the
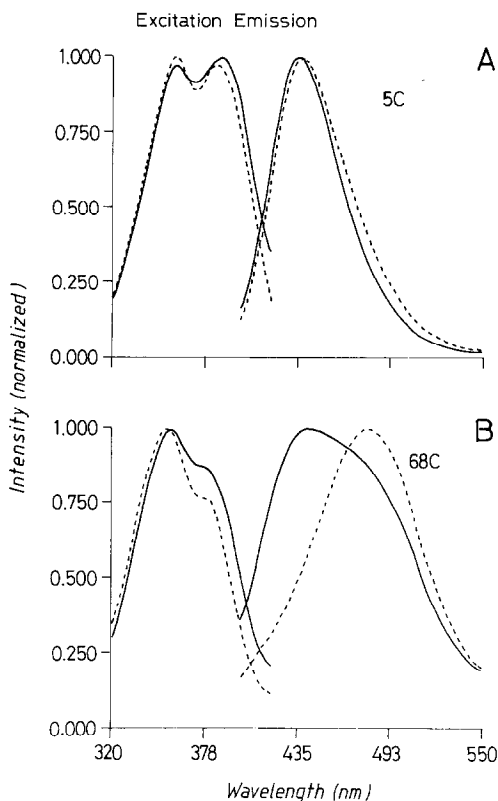
Excitation Emission

FIG. 5. Normalized Laurdan excitation and emission spectra in vesicles composed of DMPC (dotted line) and of DMPC with 30 mol% cholesterol (continuous line) at 5°C (A) and 68°C (B).

limiting GP values for the gel and the liquid–crystalline phase were 0.65 and 0.10, at 5 and 68°C, respectively, and these values were used to determine the fractional intensity of the two phases in cell membranes. Laurdan excitation and emission spectra in DMPC vesicles with 30 mol% cholesterol at 5 and 68°C are reported in Fig. 5. The spectra obtained in DMPC vesicles in the absence of cholesterol are also reported for comparison. At 5°C, in the presence of 30 mol% cholesterol, a 5-nm blue shift of the emission can be observed when compared to the spectra obtained in pure DMPC. At 68°C and in pure DMPC vesicles, the Laurdan emission maximum is at 480 nm, while in the same phospholipid with 30 mol% cholesterol a bluer emission spectrum was obtained, with the maximum intensity at 443 nm.

Laurdan GP values were measured in K562 cells as a function of time after the medium renewal using 340 nm excitation and 435 and 490 nm emission, at 20 and 37°C. The results are reported in Fig. 6A. We can observe that during cell growth, when cells progressively approach confluency, the GP value increases at both 20 and 37°C.

Using the previously determined Laurdan GP_g and GP_l values in DMPC vesicles with 30 mol% cholesterol and using the additivity rule that holds for the GP [22] (Eq. 2), we calculated the relative intensity associated with the two phases in growing and confluent cells. The

results are reported in Fig. 6B. A progressive decrease of the fraction of the liquid–crystalline phase can be observed during 5 days after the medium renewal, both at 20 and 37°C. The decrease is more evident at 20°C (Fig. 6C).

The same experiment was performed using aged K562 cells from the same clone but subcultured for several months. Also in this case we observed the increase in Laurdan GP as a function of time after the medium renewal but the percentage variation was smaller and the GP values were generally higher. In Fig. 6B the fraction of liquid–crystalline phase obtained from the Laurdan GP values of these aged cells is reported for measurements at 20°C.

More information about the properties of our cell membrane systems can be obtained by observation of the GP excitation spectrum. In a previous work [22], some characteristic features of GP excitation spectra have been reported for phospholipid vesicles in different phase states, which depend upon the relation between particular properties of the excited Laurdan molecule, such as its fluorescence lifetime and the kinetics of its dipolar relaxation. A wavelength dependence of the GP value on the excitation wavelength was observed for vesicles in which the two phases coexist, and in which only the liquid–crystalline phase is present but the relaxation phenomenon occurs. In the present work, excitation GP spectra were obtained by using emission at 435 and 490 nm.

The GP excitation spectra obtained in K562 cells are reported in Fig. 7 for measurements at 37°C, 24 h after the medium renewal. The experiments were performed on K562 cells subcultured for 3 weeks (young cells) and for 3 months (aged cells). GP excitation spectra show a wavelength dependence in both growth conditions, with a value that decreases as the wavelength increases.

DISCUSSION

In order to obtain reproducible results using Laurdan in studies of living cell membranes, particular attention must be paid to the cell labeling procedure. Since Laurdan is barely fluorescent in aqueous environments, a progressive increase of the probe concentration in cell membranes can be detected by the increase of fluorescence intensity with the time of incubation in the labeling buffer. Laurdan passively partitions into membranes and, in living cells, undergoes active compartimentalization, as demonstrated by parallel experiments using living and fixed cells. Given the amphiphilic character of the Laurdan molecule, the compartimentalization of the probe in cytoplasmic organelles most likely results from pinocytotic processes, with a mechanism similar to that reported for TMA–DPH [25, 26]. During the processes of partition and compartimentalization, the Laurdan emission shape and GP value change as a function of time of incubation in the labeling buffer, up
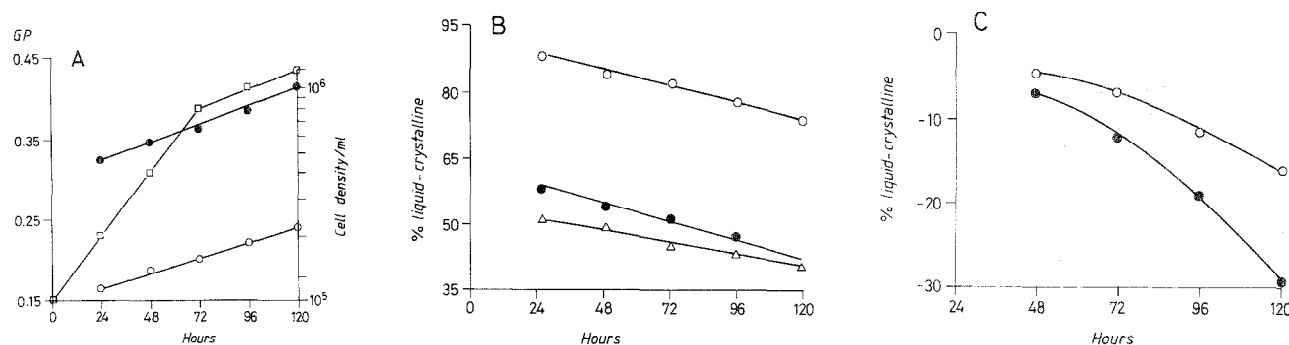
FIG. 6. (A) Laurdan GP values in K562 cells at 20°C (●) and 37°C (○) as a function of time after medium renewal. Data represent the mean of three independent experiments with SD ≤ 0.003. Also plotted is the number of cells/ml as a function of time (□). (B) Fractional intensity of the liquid–crystalline phase in K562 cell membranes obtained from Laurdan GP values at 20°C (●) and 37°C (○) as a function of time after the medium renewal. The same data are reported for "aged" K562 cells at 20°C (△). (C) Percentage decrease of the fractional intensity of liquid–crystalline phase in K562 cell membranes at 20°C (●) and 37°C (○) obtained from Laurdan GP values during the first 5 days after the medium renewal.

to 2 h, the maximum time of our observations. In preliminary experiments using fluorescence microscopy, a progressive concentration of fluorescence in cell vacuoles has been observed (not shown). These are probably the compartments in which the probe is going to be accumulated and catabolized. The high concentration of Laurdan in these organelles can explain the reduction of the total emission intensity observed in unfixed and washed cells (Fig. 2). In living cells, the probe compartimentalization continues after the removal of the labeling buffer. Nevertheless, either because the membrane composition of these compartments is the same as that of the other labeled cell membranes or because of the progressive self-quenching of concentrated Laurdan, the GP value is no longer affected by this process. The removal of the labeling buffer can be used to stabilize the GP value. Actually, the same GP value was obtained if the labeling buffer was removed after 10, 20, or 30 min of incubation, both in living and fixed cells.

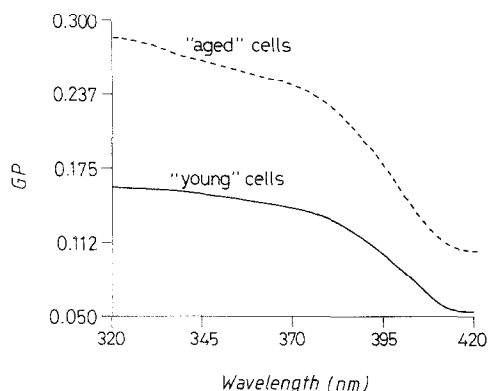A 5-nm blue shift of the Laurdan emission maximum

FIG. 7. Laurdan excitation GP spectra in K562 clone S cells at 37°C, after 24 h from the medium renewal. Before measurement, cells were subcultured for 3 weeks (continuous line) or for 3 months (dotted line). Excitation GP spectra were obtained using fixed emission wavelengths at 435 and 490 nm.

in K562 cell membranes with respect to phospholipid vesicles has been observed. The maximum emission wavelength in gel phase phospholipid vesicles is at 440 nm and is progressively red shifted when phospholipids undergo the phase transition, to a maximum at 490 nm in the liquid–crystalline phase [19–22]. In cell membranes the Laurdan maximum emission wavelength is at 435 nm, at both 20 and 37°C. From experiments performed in phospholipid vesicles containing 30 mol% cholesterol we observed a corresponding blue shift in the Laurdan emission maximum and we concluded that the typical emission maximum at 435 nm observed in cell membranes is due to the presence of cholesterol. We must point out that Laurdan can also label some membrane proteins, as we observed using a solution of bovine serum albumin and the same procedure used to label cell membranes (not shown). However, since in BSA the emission intensity was relatively low, with the maximum at 457 nm, we do not attribute the observed blue shifted maximum at 435 nm to the labeling of membrane proteins.

In a previous work, characteristic Laurdan GP values have been determined for phospholipid vesicles in the gel phase and in the liquid–crystalline phase, regardless to the phospholipid composition and pH value [22]. As already mentioned, the values were $GP_g = 0.6$ and $GP_l = -0.2$ for the gel phase and for the liquid–crystalline phase, respectively. These determinations were performed in phospholipid vesicles varying in their acyl residues and polar heads, at pH values from 4 to 10, but in the absence of cholesterol. In DMPC vesicles, in the presence of 30 mol% cholesterol and at 5°C, we obtained $GP_g = 0.65$. At 68°C, the maximum temperature of our measurements, we obtained $GP_l = 0.10$. We chose the latter value as characteristic of the liquid–crystalline phase of membranes in the presence of 30 mol% cholesterol. Our choice was arbitrary, due to the lack of a well-defined liquid–crystalline phase in the presence of cholesterol [27] and to a possible further decrease in GP

value at higher temperatures. Our choice was based on two considerations. First, 68°C is a temperature 45°C higher than the DMPC transition midpoint in the absence of cholesterol. At this temperature and with 30 mol% cholesterol, the liquid-disordered and liquid-ordered phases of membrane lipids should coalesce [27]. Second, by measuring the Laurdan GP value in K562 cell membranes at 70°C we obtained a value of 0.11. This value has to be higher than the chosen GP_l value, due to the presence of structural components in cell membranes, such as proteins, but must also be close to the GP_l value. Of course, at 70°C the GP_l value has no biological meaning.

The Laurdan GP values obtained in K562 membranes are near 0.4 at 20°C and near 0.2 at 37°C and are indicative of a phase state intermediate between gel and liquid-crystalline. By using the additivity rule that holds for polarization, these GP values can be resolved into their fractional intensities associated with the gel and liquid-crystalline phases. The fraction of liquid-crystalline phase, averaged over 5 days after the medium renewal, was 50% at 20°C and 81% at 37°C.

The possibility of resolving the GP value into two components representative of the two lipid phases is not a final proof of the coexistence of separate domains in membranes. Information on the quantum yield of Laurdan or time-resolved experiments are needed [21, 22]. Nevertheless, as extensively discussed in a previous work [22], some information about the heterogeneity of the Laurdan environment can be obtained by the dependence of the GP value on the excitation wavelength. The Laurdan GP value can vary with the excitation wavelength because of both the dipolar relaxation phenomenon and the interconversion of lipids between two coexisting phases. In the pure gel phase of phospholipids, no relaxation occurs and no dependence of GP value on excitation wavelength is observed. In the case of K562 cell membranes, a GP value decrease is obtained as the excitation wavelength increases. This behavior can indicate the presence of both a heterogeneous environment of Laurdan molecules and a homogeneous liquid-crystalline phase in the presence of dipolar relaxation. While proceeding in a more detailed understanding of Laurdan photophysics we used GP values to derive the relative fractional intensities of the gel and liquid-crystalline phases.

In Fig. 6 we report the Laurdan GP value in K562 membranes as a function of time after the growth medium renewal, i.e., as a function of cell density. As the cells approach confluence, the relative fraction of the liquid-crystalline phase decreases, from 58 to 42% at 20°C and from 90 to 74% at 37°C, at 24 and 120 h after medium renewal, respectively. Although our experiments were performed on randomly growing cells, the percentage of mitotic cells varies with time and the observed variations can be due to differences in membrane composition and dynamics during the cell duplication

cycle. The same measurements were performed in aged cells of the same clone but routinely subcultured for a few months. The GP value increased with a similar behavior, indicating the decrease of the relative fraction of the liquid-crystalline phase as a function of time after medium renewal. Nevertheless, the absolute GP values were higher than those obtained with the same clone but of young cells. We can conclude that the observed decrease in the GP value can be related both to processes involved in the cell ageing and to the difference in membrane properties in different phases of the cell duplication cycle.

By comparing the percentage decrease of the liquid-crystalline phase at 20 and 37°C, we can observe a larger slope at 20°C. This behavior could indicate that at these temperatures cell membranes are composed of coexisting phases and that a shift toward lower temperatures of the membrane phase transition occurs with cell ageing, as a function of time after the medium renewal. At 20°C cell membranes are closer to the midpoint of their phase transition and modifications of their composition will affect to a larger extent the relative percentage of each phase. At 37°C the phase composition is closer to the pure liquid-crystalline, and modifications of membrane components will produce relatively small changes in the percentage of each phase.

Using time-resolved techniques and synthetic phospholipid vesicles labeled with Laurdan, the coexistence of different phases has been demonstrated and the kinetics of fluctuation between them has been determined [21]. These time-resolved experiments are quite long and not appropriate for investigations on living biological samples. Despite more basic knowledge of Laurdan, photophysical properties are needed to finally determine if different phospholipid phase domains coexist in cell membranes by a simple GP measurement; we think the presented results offer several points in favor of this hypothesis. Recent literature reported evidence on membrane domains coexistence using fluorescence recovery after photobleaching (FRAP) [28, 29] and fluorescence microscopy [30, 31] in various cell types. Nevertheless, not one of the quoted papers was specifically concerned with domains solely constituted by different phospholipid phase states. These phase domains can have quite small dimensions, difficult to be observed by FRAP and microscopy techniques. In this perspective, we believe the relevance of the present work to reside in the possibility of an easy, fast, sensitive, and precise technique for the detection and quantitation of phospholipid phase domains in cell membranes, with the further possibility of determining the kinetics of fluctuation between domains.

As to the sensitivity of the technique we should point out that our measurements were performed on a randomly growing cell population and larger differences in membrane properties could be observed in a synchronous population. To our knowledge, no other reports

appeared on the detection of variations of membrane properties during a few days of asynchronous growth.

As specifically concerns K562 cells, no peculiar biological indicators are known to assess the ageing of this line after a few months of subculture. So far, we cannot better define the observed modifications that we simply attribute to cell ageing. To identify the molecular origin of the observed increased rigidity of cell membranes with time, further investigations are needed. Among several reports on fluidity changes in cell membranes during different physiological and pathological events [1–6, 14–16], previous investigations reported an increased concentration of cholesterol, sphingomyelin, and saturated phospholipid acyl chains during differentiation of neuroblastoma cells [32], during a 15-day growth of myocytes [33] and an increased cholesterol concentration during keratinocyte differentiation [34, 35]. These findings could be related to the short-term ageing of K562 cells, and variation of cholesterol concentration, chemical modification of membrane components and lipids, and membrane proteins turnover could be responsible for the observed increase in Laurdan GP value. We also need to take into account that each cell type may have a specific behavior during growth and differentiation. For instance, different from the above quoted reports, a decrease in the membrane order has been found during growth and differentiation of adipocyte cells [36].

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