Use of a Fluorescent Probe to Compare the Plasma Membrane Properties in Normal and Transformed Cells. Evaluation of the Interference by Triacylglycerols and Alkyldiacylglycerols†

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ABSTRACT: The fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) was used to study the plasma membrane properties of normal and Rous sarcoma virus transformed chicken embryo fibroblasts. When measurements were made on whole cells, the fluorescence polarization of different cell preparations showed an inverse correlation with the amount of triacylglycerols and alkyldiacylglycerols present in the cells. These neutral lipids were stored as droplets within the cell and their accumulation depended on the concentration of serum in the medium, the cell density, and the time of growth. When plasma membranes were isolated that contained negligible amounts of triacylglycerols and alkyldiacylglycerols, the fluorescence polarization of DPH in the membranes was very different from the polarization of DPH in intact cells. The changes in the polarization of the probe in intact cells under different conditions were primarily reflecting the changes in the amount of triacylglycerols and alkyldiacylglycerols. DPH in plasma membranes isolated from transformed cells had a slightly higher fluorescence polarization than in membranes from normal growing cells. DPH in total lipid extracts and phospholipids prepared from the transformed cell membranes also had a higher polarization as compared with the respective extracts from normal growing cells. The higher polarization appeared to be due to increases in the concentration of phosphatidylethanolamine and 18:1 and to decreases in the amount of polyunsaturated fatty acids. Plasma membranes and total lipid extracts prepared from confluent cells had a greater increase in the polarization of DPH when compared with normal growing cells. No difference in polarization of DPH was found, however, for the phospholipids from membranes of normal growing and confluent cells. The difference in polarization for the membranes and total lipid extracts was consistent with an increase in the cholesterol content of the confluent cells as compared with normal growing cells.

Differences in the physical and biochemical characteristics of the plasma membrane between normal and transformed cells have received much attention (for a review, see Robbins & Nicolson, 1975). There have been several reports of changes in the lipid composition accompanying malignant transformation: decreased cholesterol content for mouse lymphoma cells (Inbar & Shinitzky, 1974), decreased ratio of phosphatidylcholine to phosphatidylethanolamine for Rous sarcoma virus transformed chicken embryo fibroblasts (Hale et al., 1977), and an increased ratio of oleic to arachidonic acid in rat hepatomas (Veerkamp et al., 1962), SV40-transformed WI-38 (Howard & Kritchevsky, 1969), and chicken embryo fibroblasts (Yau et al., 1976). In order to determine the role of lipids in transformation-associated membrane properties, methods were developed to manipulate the lipid composition of normal and Rous sarcoma virus infected chicken embryo fibroblasts (Hale et al., 1977).

Electron spin resonance spectroscopy has been one method used to study the physical properties of the cell membrane upon transformation. Gaffney (1975) found no difference in the motion of spin labels between normal and transformed fibroblasts while Yau et al. (1976) found a slightly decreased motion of spin labels in transformed fibroblasts. Another method used to study the properties of the cell membranes upon transformation has been the technique of fluorescence polarization (Shinitzky et al., 1971; Cogan et al., 1973). In particular, the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH)[1] has been extensively used to study the properties of liposomes and biological membranes. It has excellent spectral properties (Shinitzky & Barenholz, 1974) and partitions equally between gel and liquid-crystalline lipid phases (Leutz et al., 1976). Measurements conducted on intact cells after the addition of DPH have indicated that plasma membranes in normal lymphocytes have a much higher polarization and "microviscosity" than malignant lymphocytes (Shinitzky & Inbar, 1974), while untransformed fibroblasts have a lower polarization and "microviscosity" than transformed fibroblasts (Inbar et al., 1977; Fuchs et al., 1975). Changes in the polarization of DPH and "microviscosity" have also been reported as a function of cell density (Inbar et al., 1977). In these studies on whole cells it was assumed that the fluorescent probe was predominantly localized in the plasma membrane and, consequently, the measurements were reflecting the properties of the plasma membrane. In general, measurements made with hydrophobic probes either in whole cells or in membrane preparations do not take into account the presence of triacylglycerols and alkyldiacylglycerols.

In a previous study using DPH to determine the properties of LM cell membranes with altered lipid compositions, we found that DPH would rapidly partition into the interior of the cells (Esko et al., 1977). Not only was DPH found in intracellular membranes, but most importantly, it also partitioned into nonmembranous triacylglycerols and alkyldiacylglycerols. These neutral lipids had a polarization value very different

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[1] Abbreviations used: DPH, 1,6-diphenyl-1,3,5-hexatriene; PBS, phosphate-buffered saline; Tris, tris(hydroxymethyl)aminomethane; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SPH, sphingomyelin; PI, phosphatidylinositol; PS, phosphatidylserine; CL, cardiolipin.

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from the membranes and could significantly alter measurements made on whole cells or impure membrane preparations. The LM cells were different from most tissue culture cells in that they were grown in a chemically defined medium that contained no serum or lipid. Recently, Pagano et al. (1977) using autoradiography and Berlin & Fera (1977) using fluorescence microscopy have shown that DPH can be internalized by several different types of intact cells. On the other hand, De Laat et al. (1977) on the basis of fluorescence microscopic observations concluded that DPH is confined to the cell surface membrane of neuroblastoma cells.

In this paper we describe the effects of triacylglycerols and alkyldiacylglycerols on the polarization of DPH in normal and Rous sarcoma virus infected chicken embryo fibroblasts. The polarization of DPH and its correlation with the lipid composition of isolated plasma membranes are reported.

Materials and Methods

Cell Culture. Primary cell culture and virus infection were carried out as previously described (Hale et al., 1977). After 3 days as primary cultures, normal or Rous-infected cells were trypsinized with 0.05% crystalline trypsin (Miles Servac, Grade 1) in Tris-buffered saline (137 mM NaCl, 5.1 mM KCl, 0.7 mM Na_2HPO_4, 24.8 mM Tris, pH 7.4), and replated as secondary cultures at 1.0×10^4 cells/cm^2 for the transformed cells, 0.7×10^4 cells/cm^2 for normal growing cells, and 1.0×10^5 cells/cm^2 for the confluent cells. Because a slightly lower percentage of the transformed cells attach and grow, these plating densities result in similar cell densities between normal growing and transformed cells.

Secondary cultures were plated in Dulbecco's modified Eagle's medium supplemented with 4% calf, 1% chicken serum for both the normal growing and transformed cells, and in 1% calf, 1% chicken serum for the confluent cells. Large scale production of cells for plasma membrane isolation was performed in roller bottles; otherwise cells were grown on plastic tissue culture dishes.

Electron Microscopy. Cells were washed twice with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 12.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2) at 37 °C, scraped, and centrifuged at 2000 rpm for 5 min. Three percent glutaraldehyde in a 0.1 M phosphate buffer (pH 7.2) at 4 °C was added to the centrifuge tube and the pellet was removed. It was sliced into 1-mm^3 sections and placed into 1% osmium tetroxide for 2 h at 4 °C. The sections were rinsed and allowed to remain under 3% glutaraldehyde for 24 h.

Unstained sections were prepared by rinsing the above sections twice with phosphate buffer, and placing them in a 1% myrcene (Aldrich), 0.1% ethylgallate (ICN) in 70% ethanol solution for 2 h. These sections were rinsed with 50% ethanol and placed into 1% osmium tetroxide for 1 h, followed by dehydration in an ethanol series (10% to 100%) and embedding in Spurr's medium. This procedure specifically increases the contrast for saturated and unsaturated lipids (Wigglesworth, 1975).

Stained sections were prepared in an analogous fashion except they were placed into 20% uranyl nitrate and Reynolds lead citrate before the dehydration. A JEOL 100c transmission electron microscope was used to visualize the sections.

Lipid Analysis. The lipids in cells and isolated plasma membranes were extracted using the method of Bligh & Dyer (1959) as described by Ames (1968). Phospholipid analyses were carried out on cells that were labeled continuously with 1.0 μCi/mL of [^{32}P]phosphate beginning at plating of the primary cultures. Phospholipids were separated by two-dimensional thin-layer chromatography on silica gel G (250 μm, Analtech, Inc.) as previously described (Glaser et al., 1974). Spots were visualized by autoradiography, scraped, and counted.

Fatty acid methyl esters were prepared from phospholipids obtained after separation of the phospholipids and neutral lipids on a 2-in. unisil column (Ferguson et al., 1975). Methyl esters were prepared in sodium methoxide–methanol (Applied Science) and chromatographed isothermally at 180 °C on a 1.5 m × 0.317 cm column of cyanosilicone SP-2340 on a 80–100 mesh Chromosorb W AW (Supelco, Inc.).

Cholesterol content was determined using a column of cyanosilicone SE-30 on 80–100 mesh Chromosorb Q, essentially as described by Sokoloff & Rothblat (1972). 5α-Cholestane was used as an internal standard.

The amounts of triacylglycerols and alkyldiacylglycerols obtained after the unisil column were measured together by the procedure of Snyder & Stephens (1959). This method determines the amount of ester bond equivalents in the sample. Chicken fibroblasts have negligible amounts of sterol esters (determined by thin-layer chromatography) which would interfere with the assay. Alkyldiacylglycerols comprise approximately 20–30% of the total amount of ester equivalents present in the neutral lipids. Total phospholipid phosphate was determined by Ames ashing (Ames & Dubin, 1960) or alternatively, by the ester assay on the phospholipid fraction from the unisil column assuming two ester equivalents per mole of phospholipid.

Plasma Membrane Isolation. The procedure for the isolation of plasma membranes has been previously described (Esko et al., 1977) and was used with one minor modification. After the nuclei were removed by shearing and low speed centrifugation, the supernatant was centrifuged at 18 000g for 20 min, instead of 48 000g for 60 min. This was found to pellet the mitochondrial and plasma membrane fractions while leaving substantial amounts of the microsomal fraction in the supernatant. The plasma membrane was then separated from the mitochondria and remaining microsomes by a discontinuous sucrose gradient (Esko et al., 1977). The purification of the plasma membrane consistently ranged from 4- to 6-fold over the particulate homogenate as measured by the specific activity of the ouabain-sensitive (Na^+/K^+)ATPase (Esko et al., 1977) with negligible succinate-dependent cytochrome c reductase (mitochondrial marker) and 0.8- to 1.5-fold purification of TPNH-dependent cytochrome c reductase (microsomal marker) (Sottocasa et al., 1967). After the purification, the content of triacylglycerols and alkyldiacylglycerols was less than 2% of the phospholipids. Protein was determined using bovine serum albumin as a standard (Lowry et al., 1951).

Fluorescence Techniques. Polarization measurements of whole cells and membranes were carried out as described previously (Esko et al., 1977) and are based on the methods of Shinitzky & Barenholz (1974) and Shinitzky & Inbar (1974). Cells were washed twice with PBS[1] at 37 °C, then scraped, resuspended, and counted in a Coulter particle counter. Cells (1×10^5) were incubated at 37 °C in 2 mL of a 2 μM aqueous dispersion of DPH (Eastman Organic Chemicals; twice recrystallized from hexane) in PBS for 30 min. The cells were then used directly for polarization measurements. Plasma membrane polarization measurements were performed by incubating 50 μg of protein of the purified membrane preparation in 2 mL of the DPH solution. Changing the labeling conditions by altering the DPH concentration or the temperature had no effect on the polarization values (Esko et al., 1977). In all cases energy transfer was negligible since over the course of the incubation there was an increase in the fluorescence intensity, but the polarization remained constant.

Since the polarization reached a constant value by the first reading (2 min), DPH must have equilibrated fairly rapidly within the cell after it was taken up from the medium.

Lipid dispersions for fluorescence measurements were obtained by the method of Bangham et al. (1965). DPH was added to 30 nmol of extracted lipid phosphate in $CHCl_3$ to give a 1:1000 mol ratio of DPH to lipid phosphate. The lipid–DPH mixture was dried under a stream of nitrogen and then placed in a vacuum desiccator overnight. The lipid was dispersed into 2.5 mL of PBS and incubated at 55 °C under nitrogen for 60 min with periodic vortexing. Scattered light was reduced to less than 2% by use of a 2 M $NaNO_2$ solution and 3-72 cut-off filters (Shinitzky et al., 1971). Each point represents the average of 50 measurements and the error in the polarization values obtained in this manner was less than ±0.002.

Lifetimes were measured directly on a cross-correlation phase fluorometer described by Spencer & Weber (1969), with updated electronics by SLM Instruments, Inc. As was observed previously, lifetimes deduced from measurements of the fluorescence intensity did not always give reliable values (Esko et al., 1977). Light scattering by the samples did not significantly change the observed fluorescence lifetimes and thus a correction for light scattering was not required. Lifetimes were the average values calculated at 10 MHz, from phase (τ_{phase}) and modulation (τ_{mod}). The difference between τ_{phase} and τ_{mod} was approximately 1 ns which does not indicate a large heterogeneity in the probe environment (Weber et al., 1976). Consequently, the data were treated as if the probe was in a single environment.

Rotational relaxation times were calculated from the measured fluorescence polarization and lifetime values using the Perrin equation (Weber, 1953):

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_0} - \frac{1}{3}\right)\left(1 + \frac{3\tau}{\bar{\rho}}\right)$$

where P is the polarization, τ is the lifetime of the excited state, and ρ is the rotational relaxation time.[2] $P_0 = 0.460$ for DPH (Shinitzky & Inbar, 1974) and is the polarization in the absence of rotational motion. This equation applies to nonspherical fluorophores when the absorption and emission oscillators are parallel (Shinitzky et al., 1971). This is true for DPH when it is excited in the last absorption band. In this case the rates of rotation in the plane and out of the plane of the molecule contribute equally to the observed depolarization. $\bar{\rho}$ represents the harmonic mean of the rotational relaxation times for the in-plane and out-of-plane motions.

Results

It has been known for some time that animal cells growing in a medium containing serum are capable of taking up fatty acids and using them for the synthesis of phospholipids and triacylglycerols (see Rothblat & Kritchevsky, 1967). As the concentration of exogenous fatty acids is increased, progressively more triacylglycerols are formed and stored in the cells. The rate and extent to which Rous sarcoma virus infected cells (Figure 1A) and normal growing cells (Figure 1B) acquire and subsequently lose triacylglycerols and alkyldiacylglycerols were similar, both reaching a maximum accumulation approximately 24 h after plating. On the other hand, normal cells, plated at a high density and lower serum concentration so that they were confluent and density inhibited in growth, acquired substantially reduced amounts of these neutral lipids, with a maximum accumulation approximately 10 h after plating (Figure 1C).

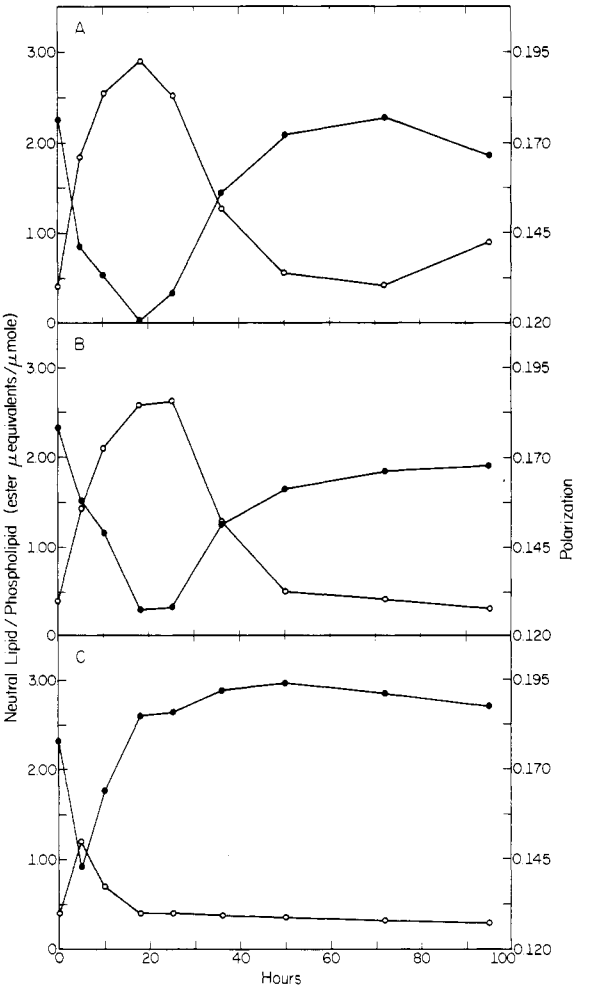

FIGURE 1: Whole cell fluorescence polarization at 37 °C (●) and accumulation of triacylglycerols and alkyldiacylglycerols (○) in (A) transformed, (B) normal growing, and (C) confluent cells. Fluorescence polarization and neutral lipid assays were performed as described in Materials and Methods.

When the cells were labeled with DPH, the fluorescence polarization showed a strong inverse correlation with the content of triacylglycerols and alkyldiacylglycerols in all three cases (Figure 1). The polarization was initially high and decreased until it reached a minimum value at the same time the amount of triacylglycerols and alkyldiacylglycerols was at a maximum. As the amount of triacylglycerols and alkyldiacylglycerols declined the polarization increased.

For 30 h, there was very little difference in the polarization for transformed and normal growing cells. At later times, however, the transformed cells had a higher polarization, although both had similar amounts of neutral lipids. The confluent cells had the same fluorescence polarization as did the normal growing cells up to 10 h after plating, after which there was a much larger increase in the polarization of the confluent cells.

Transmission electron micrographs of transformed cells containing high low levels of triacylglycerols and alkyldiacylglycerols were taken (Figure 2). The cells were prepared in two ways, either stained with heavy metals (Figures 2C and 2D), or unstained (Figures 2A and 2B). These two techniques clearly distinguished these lipids as electron dense bodies within the cytoplasm of the cells. Cells with a low content of these neutral lipids had very few dense bodies (Figure 2A). In

[2] The rotational relaxation time is the time it takes for molecules to rotate through an angle θ, such that $\overline{\cos\theta} = e^{-1}$.

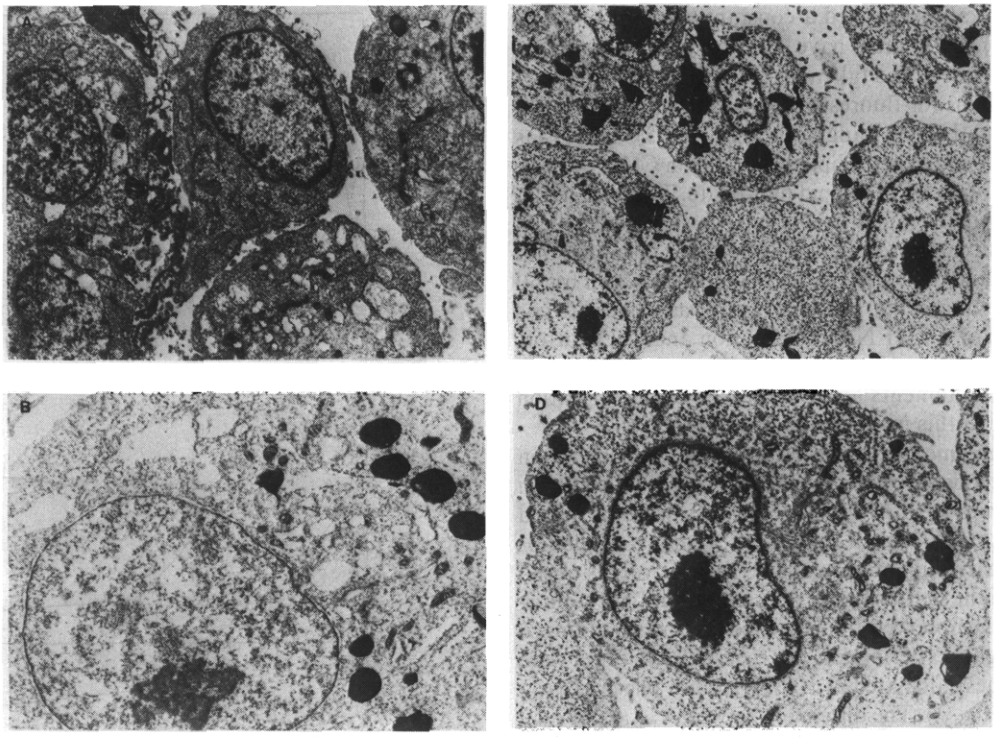

FIGURE 2: Transmission electron micrographs of transformed cells containing (A) low levels of neutral lipid droplets and (B, C, and D) high levels of neutral lipid droplets. Sections were either stained with heavy metals or left unstained as described in Materials and Methods. (A) Unstained before plating (3630×); (B) unstained 24 h after plating (5445×); (C and D) stained 24 h after plating (2310× and 5225×, respectively).

comparison, cells with a high neutral lipid content had many distinct and large dense bodies (Figure 2C). The Wigglesworth procedure specifically increases the contrast of saturated and unsaturated lipids (Wigglesworth, 1975), although both methods clearly showed the lipid droplets. A similar increase in neutral lipid droplets of normal growing cells was seen in electron micrographs in which the amount of triacylglycerols and alkyldiacylglycerols was increased as measured by the spectrophotometric assay (data not shown).

One of the largest changes in the polarization of DPH in whole cells occurred when the medium was charged after 20 h of growth. Twenty-four hours later the polarization at 37 °C decreased from 0.150 to 0.115 and the amount of triacylglycerols and alkyldiacylglycerols had increased over threefold so that there were more of these neutral lipids than phospholipids on a mole basis (Table I). When the cells were lysed and centrifuged at high speed, the triacylglycerols and alkyldiacylglycerols were generally concentrated near the top of the centrifuge tube. This supernatant had a very low polarization and could account for the whole cell polarization being decreased relative to the isolated membranes. In comparing the polarization values for the isolated membranes or comparing the total lipid extracts between the normal growing cells and the cells after the medium was changed, no differences were apparent. When the total lipid extract was passed over a unisil column to remove cholesterol, the phospholipids also had similar polarization values for the two cell types. Consistent with the similar polarization values, there were no significant differences in the cholesterol to phospholipid ratio, phospholipid composition, or the fatty acid composition between the membranes from these cells (data not shown).

When the polarization of DPH was measured in intact confluent cells the values were higher than for normal growing cells after 45 h which correlated with their lower content of triacylglycerols and alkyldiacylglycerols (Figure 1 and Table I). Isolated plasma membranes as well as the total lipid extract from the confluent cells had higher polarization values than the normal growing cells, but the phospholipids were identical (Table I).

The phospholipid compositions of whole cells or of the plasma membranes were essentially identical for normal growing and confluent cells (Table II). The fatty acid compositions also were similar except for a small decrease in the percentage of 18:2 in the confluent cells (Table III). The cholesterol to phospholipid ratio, however, was significantly increased for the confluent cells in both the whole cells and the plasma membranes (Table IV). This appears to account for the increased polarization of DPH in the membranes and the total lipid extract from the confluent cells.

Fluorescence polarization measurements of DPH in Rous sarcoma virus transformed and normal growing whole cells and plasma membranes are shown in Table V. The polarization of DPH in intact transformed cells was higher than for normal growing cells, even though the content of triacylglycerols and alkyldiacylglycerols was similar. Isolated plasma membranes, total lipids, and phospholipids from the transformed cells also had higher polarization values than did the normal growing cells. In this case, there should be no change in the cholesterol content but rather a change in the phospholipid or fatty acid composition. The data in Table IV show that there was little change in the cholesterol to phospholipid ratio on transformation, but there was an increase in the phosphatidylethanolamine to phosphatidylcholine ratio as observed previously (Hale et al., 1977; Table II). There was also a decrease in the percent of polyunsaturated fatty acids and an increase in the ratio of 18:1 to 20:4 in the membrane phospholipids (Hale et al., 1977; Yau et al., 1976; Table III). Isolated plasma membranes from normal growing and transformed cells both had similar ratios of phospholipid to protein (approximately 500 nmol of phospholipid phosphate per mg of protein).

Absolute values for the polarization of DPH or the phospholipid composition for a given cell or membrane varied

TABLE I: Fluorescence Polarization of DPH in Fractions Obtained from Normal Growing Cells, Normal Growing Cells after Medium Change, and Confluent Cells.[a]

Fraction	Polarization 37 °C	25 °C	10 °C	Neutral lipid/phospholipid[b]
	Normal Growing Cells			
Whole cells	0.150	0.182	0.239	1.30
48 000*g* supernatant[c]	0.111	0.133	0.196	
Plasma membrane	0.220	0.263	0.315	
Total lipids[d]	0.239	0.282	0.330	
Phospholipids	0.140	0.174	0.236	
	Normal Growing Cells after Medium Change[e]			
Whole cells	0.115	0.151	0.213	4.27
48 000*g* supernatant[c]	0.105	0.120	0.178	
Plasma membrane	0.219	0.264	0.317	
Total lipids[d]	0.239	0.284	0.332	
Phospholipids	0.142	0.176	0.237	
	Confluent Cells			
Whole cells	0.188	0.229	0.282	0.38
Plasma membrane	0.246	0.285	0.331	
Total lipids[d]	0.264	0.300	0.346	
Phospholipids	0.141	0.178	0.242	

[a] Fluorescence polarization measurements, cell culture, and plasma membrane isolation were carried out as described in Materials and Methods. Cells were harvested 45 h after plating. A second experiment on these three types of cells gave essentially identical results. [b] The ratio of neutral lipid ester μequiv to μmol of lipid phosphate. [c] The 18 000*g* supernatant which contained the majority of neutral lipids was centrifuged at 48 000*g* for 1 h to remove microsomes. [d] Total lipids from the plasma membranes consisted of phospholipids and cholesterol. The DPH to lipid phosphate ratio was 1:1000. [e] After 20 h of growth, normal cells had their medium removed and replaced with fresh medium containing 8% calf and 2% chicken serum. Plasma membrane isolation was performed 25 h later.

TABLE II: Phospholipid Composition of Whole Cells and Plasma Membranes from Transformed, Normal Growing, and Confluent Cells.[a]

	Phospholipid composition (%) PE	PC	PI + PS	SPH	CL	Others
	Transformed Cells					
Whole cell	32.6	40.5	13.3	7.8	2.3	3.4
Plasma membrane	34.5	35.0	14.3	12.5	0.5	3.2
	Normal Growing Cells					
Whole cell	26.4	44.8	14.7	8.3	2.6	3.2
Plasma membrane	26.9	40.5	14.4	14.1	0.6	3.5
	Confluent Cells					
Whole cell	23.6	46.7	13.5	10.6	1.8	3.8
Plasma membrane	28.2	40.4	11.0	18.5	0.3	1.6

[a] The determination of the phospholipid composition and the plasma membrane isolation were carried out as described in Materials and Methods. Cells were harvested 45 h after plating. In a second experiment similar compositions were found for normal and transformed cell plasma membranes with PE increasing from 30.8% to 34.4% and PC decreasing from 36.4% to 34.5% on transformation.

slightly between different experiments. However, the increase in the polarization of DPH in transformed cell membranes as compared with normal growing cell membranes, for example, always increased in the same manner (in a total of five experiments). In each experiment the growth, membrane isolation, and measurements were carried out simultaneously on parallel cultures of cells to ensure comparable conditions as much as possible.

A change in the polarization of DPH at a given temperature can be caused either by a change in the rate of rotation of DPH due to an altered viscosity of its environment or by a change in the lifetime of the excited state. The lifetime of DPH decreased slightly in going from plasma membranes to total lipid extracts to phospholipids (Table VI). However, the lifetimes of DPH were quite similar for a given fraction regardless of whether the fraction was obtained from normal growing or transformed cells. Consequently, the rotational relaxation times showed the same trends as the polarization when comparing plasma membranes, total lipids, or phospholipids between normal growing and transformed cells. The temperature dependencies of the rotational relaxation times were also similar for the three fractions.

Discussion

Fluorescent probes are widely used to study the properties of membranes. Because of the sensitivity of fluorescence measurements this method is particularly applicable to the study of membranes from tissue culture cells or other types of cells where obtaining a reasonable quantity of isolated membranes is often a problem. The results presented in this paper point out a problem in using a hydrophobic probe to determine the properties of membranes in intact cells or in crude membrane preparations. Cells in culture will accumulate triacylglycerols and alkyldiacylglycerols to substantial levels in a manner that depends on the serum concentration, time after plating, and cell density. When DPH was added to whole cells,

TABLE III: Fatty Acid Composition of Whole Cells and Plasma Membranes from Transformed, Normal Growing, and Confluent Cells.[a]

	Fatty acid composition (%)													18:1/ 20:4	% saturated fatty acids	% polyunsaturated fatty acids
	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:3	20:4	22:4	22:5	22:6	Other			
Transformed Cells																
Whole cell	14.1	2.5	19.8	23.5	13.6	0.4	1.0	4.0	10.5	2.9	2.8	1.9	3.0	2.2	34.9	36.1
Plasma membrane	14.5	2.3	21.3	22.1	11.9	0.4	1.0	4.5	9.4	4.4	2.9	2.0	3.3	2.4	36.8	36.5
Normal Growing Cells																
Whole cell	11.7	1.0	24.0	19.0	10.6	0.3	0.6	2.5	16.8	4.0	5.8	2.3	1.5	1.1	36.3	42.2
Plasma membrane	12.6	1.2	23.5	18.1	10.1	0.3	0.4	4.2	15.7	3.8	5.6	2.9	1.6	1.1	36.5	42.6
Confluent Cells																
Whole cell	14.1	1.2	22.3	19.8	5.7	0.2	0.5	3.3	19.0	5.9	2.8	3.6	1.6	1.0	36.9	40.5
Plasma membrane	16.5	1.4	22.4	18.6	4.6	0.1	0.4	3.3	17.8	6.4	3.0	4.2	1.3	1.0	39.3	39.4

[a] Plasma membrane isolations and fatty acid analysis were carried out as described in Materials and Methods. Cells were harvested 45 h after plating. In a second experiment similar results were obtained with transformed cell plasma membranes having a relative decrease in the percentage of 20:4 and polyunsaturated fatty acids (8% and 9%, respectively) and a relative increase in the percentage of 18:1 (7%) compared with normal growing cell plasma membranes. Confluent cell plasma membranes had a 4% decrease in the percentage of 18:2 compared with normal cell plasma membranes.

TABLE IV: Cholesterol Content of Whole Cells and Plasma Membranes from Transformed, Normal Growing, and Confluent Cells.[a]

	Cholesterol/phospholipid (mole ratio)		
	Transformed cells	Normal growing cells	Confluent cells
Experiment no. 1			
Whole cells	0.29	0.28	0.35
Plasma membranes	0.52	0.48	0.60
Experiment no. 2			
Whole cells	0.27	0.23	0.38
Plasma membranes	0.47	0.52	0.61

[a] The cholesterol content was determined as described in Materials and Methods. The conditions were the same as those in Table II.

it rapidly partitioned into these neutral lipid droplets and a strong correlation was observed between changes in polarization and changes in the amount of triacylglycerols and alkyldiacylglycerols. Consequently, polarization measurements can give very different results simply by growing a given cell type under different conditions or harvesting at different times even though no change is occurring in the cell membranes. Care must also be taken during the isolation of membranes since many procedures result in membrane vesicles that can trap variable amounts of triacylglycerols and alkyldiacylglycerols. It is likely that this is a general problem for any hydrophobic probe that is used for fluorescence or electron spin resonance studies.

A comparison of isolated plasma membranes revealed differences in the fluorescence polarization measured by DPH in transformed, normal growing, and confluent cells. In order to understand the basis for these differences, measurements were made on total lipid extracts and phospholipids from the membranes. In each case the total lipid extracts gave slightly higher polarization values than the plasma membranes from which they were derived. This difference might be due to the influence of the membrane proteins. Alternatively, it might be generated in the lipid extraction or in the gross structural organization of the lipid dispersions as compared with the plasma membrane. The total lipid extracts of LM cells, for example, can give lower (Esko et al., 1977) or higher (Barenholz et al., 1976) polarization values as compared with the membranes. When cholesterol was removed from the total lipid extracts, the remaining phospholipids had a decreased polarization as would be expected (see Shinitzky & Inbar, 1974).

Isolated membranes and total lipid extracts from confluent cells had a higher polarization as compared with normal growing cells, while no difference was found for the phospholipids. This is consistent with the increased cholesterol to phospholipid ratio in confluent cells, while there was no significant change in the phospholipid or fatty acid composition.

In the case of transformed cells the isolated membranes, total lipid extracts, and phospholipids all showed the same relative increase in the polarization of DPH as compared with normal growing cells. These data can be accounted for by the changes observed in the fatty acid and phospholipid compositions. Transformed cells had an increase in the ratio of phosphatidylethanolamine to phosphatidylcholine which produces an increase in the polarization as measured by DPH (Esko et al., 1977). Transformed cells also had an increase in 18:1 and a decrease in long-chain polyunsaturated fatty acids which would be expected to produce an increase in the polarization.

In order to understand the "fluidity" or viscosity of biological membranes, it is important to be able to measure directly the motion of molecules in a sensitive and quantitative way. Fluorescence polarization provides a method for obtaining this type of information. Fluorescence polarization, however, is not only a function of the rate of rotation of the fluorophore but also of the lifetime of the excited state. The Perrin equation can be used to relate the polarization and lifetime to give a "mi-

TABLE V: Fluorescence Polarization of DPH in Fractions Obtained from Transformed and Normal Growing cells.[a]

Fraction	Polarization 37 °C	Polarization 25 °C	Polarization 10 °C	Neutral lipid/phospholipid[b]
		Transformed Cells		
Whole cells	0.164	0.205	0.257	0.60
Plasma membrane	0.239	0.283	0.336	
Total lipids	0.251	0.293	0.343	
Phospholipids	0.148	0.198	0.257	
		Normal Growing Cells		
Whole cells	0.157	0.201	0.257	0.71
Plasma membrane	0.229	0.273	0.328	
Total lipids	0.240	0.284	0.337	
Phospholipids	0.140	0.189	0.253	
		Polarization Differences[c]		
Plasma membrane	0.011 ± 0.003	0.010 ± 0.004	0.009 ± 0.004	
Total lipids	0.013 ± 0.005	0.013 ± 0.004	0.009 ± 0.003	
Phospholipids	0.012 ± 0.006	0.015 ± 0.006	0.006 ± 0.004	

[a] Conditions were the same as those in Table I. [b] The ratio of neutral lipid ester μequiv to μmol of lipid phosphate. [c] The polarization of DPH in fractions from transformed cells minus the values for normal growing cells. The standard deviations, $\sigma = [\Sigma(X - \bar{X})^2/(n-1)]^{1/2}$, were calculated for the differences in polarization from five separate paired experiments.

TABLE VI: Fluorescence Lifetimes and Rotational Relaxation Times for DPH in Fractions Obtained from Transformed and Normal Growing Cells.[a]

	τ (ns) 37 °C	τ (ns) 25 °C	τ (ns) 10 °C	$\bar{\rho}$ (ns) 37 °C	$\bar{\rho}$ (ns) 25 °C	$\bar{\rho}$ (ns) 10 °C
			Transformed Cells			
Plasma membrane	10.0	10.4	10.6	27.5	42.2	73.0
Total lipids	9.6	10.2	10.4	29.3	45.5	77.4
Phospholipids	8.9	9.6	10.1	10.7	18.4	32.5
			Normal Growing Cells			
Plasma membrane	10.1	10.6	10.8	25.4	39.3	68.2
Total lipids	9.1	9.8	10.4	25.2	40.2	72.4
Phospholipids	8.7	9.2	10.2	9.7	16.3	31.7

[a] The fluorescence lifetimes were determined on the same fractions used in Table V. The values of the polarization given in Table V along with the lifetime values were used to calculate $\bar{\rho}$ as described in Materials and Methods.

croviscosity" which is a more relevant parameter. The precise motion undergone by DPH in membranes is not known (as is the case in general) and some assumptions necessary for this calculation have been questioned (Chen et al., 1977; Kawato et al., 1977). One assumption that may not be valid is that the motion of DPH in a reference oil is not equivalent to the motion of DPH in a bilayer (Dale et al., 1977). The calculation of a purely phenomenological parameter, the rotational relaxation time, rather than a "microviscosity" by means of the Perrin equation seems a more appropriate way to characterize the motions in membranes.

One objective of this work was to determine whether there were any differences in the "fluidity" of the plasma membranes from transformed, normal growing, and confluent cells. In this respect, it seems reasonable to compare the changes in rotational relaxation (or polarization if the lifetimes are constant) for DPH in similar membrane preparations. The results show approximately an 8% increase at 37 °C in the rotational relaxation time for transformed cell membranes as compared with normal growing cell membranes. The change was larger for membranes from confluent cells as compared with membranes from normal growing cells (approximately 20% at 37 °C assuming constant lifetimes). Since the measurements reflect an average over all environments of DPH, it is possible that the changes are more pronounced in critical domains or localized regions of the membrane. The exact motions DPH undergo and the physiological significance of the changes that were observed remain to be determined. In future experiments using hydrophobic probes to study membrane properties the presence of triacylglycerols and alkyldiacylglycerols have to be considered.

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