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Effects of culture and incubation conditions on membrane fluidity in monolayers of cultured cells measured as fluorescence anisotropy using trimethylammoniumdiphenylhexatriene (TMA-DPH)

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Membrane fluidity of coverslip attached living cells was measured as fluorescence anisotropy using 5 μM trimethylammoniumdiphenylhexatriene (TMA-DPH) as fluorescent probe. Fluorescence anisotropy is inversely related to membrane fluidity. Cells were grown on glass coverslips that were inserted and directly incubated in quarz cuvettes. The coverslips were fixed with special holders at an angle of 30° in respect to the incident light. Effects of incubation temperature, of cell growth and densities and of the ionic and nonionic composition of the incubation medium on membrane fluorescence anisotropy were measured. Membranes of growing cells were more fluid than those of stationary cells, while cell densities had no effect except at very low cell numbers. Calcium concentrations increasing from 0 to 8 mmol/l in the incubation medium proportionally decreased membrane fluidity. Hypotonicity of the incubation media increased membrane fluidity while hypertonicity compared to normotonicity had no effect. Differentiated human fibroblasts from different origins exhibited similar membrane fluidities. They were, however, different from those of rat cells. Membrane fluidity of rat brain tumor cells increased with age in culture while membrane fluidity of primary differentiating rat brain cells decreased in with age in culture. Measurement of fluorescence anisotropy in living cells attached to glass coverslips is a convenient tool to study effects of culture – as well as of environmental – conditions on membrane fluidity.

Introduction

Membrane fluidity is a biophysical property of the membrane quantitatively expressing the mobility and the rate of rotational motions of membrane lipid molecules. Those parameters can be investigated by physical methods such as electron spin resonance, magnetic resonance spectroscopy and fluorescence anisotropy. Determination of steady-state fluorescence anisotropy is widely used to investigate biological systems because of its simplicity and sensitivity.

Membrane fluidity appears to change during development [1], aging [2] and drug therapy of cultured cells [3,4]. Changes in membrane fluidity are known to be linked to alterations in physiological processes of the cell membrane like carrier-mediated transport, activities of membrane bound enzymes, receptor binding, phagocytosis, endocytosis, depolarization dependent exocytosis, cytotoxicity and cell growth [5–9]. Alterations in membrane fluidity may even represent a possible parameter in the evaluation of malignancy [10].

Fluorescence anisotropy can be measured by introducing a fluorescent probe, in our case 1-[4-(trimethylamino)-phenyl]-6-phenylhexa-1,3,5-triene (TMA-DPH), into the phospholipid bilayer. This fluorescent substance is a cationic analogue of diphenylhexatriene (DPH). The cationic charge ensures that the probe is anchored at the water/lipid interface, most likely with the DPH-moiety intercalated between the upper portions of the fatty acyl chains [11]. Relatively little work

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has been done on whole cells with this marker and most experiments have been performed in suspensions of scraped or trypsinized cultured cells [12].

Cultures in situ growing on glass surfaces have been used for fluorescence anisotropy measurements by Sumbilla and Lakowicz [13] and others [14–18]. To our knowledge no work has been published on the effects of tissue culture- and of incubation-conditions on the anisotropy measurements. We now report on effects of such conditions on fluorescence anisotropy measurements using different glass attached monolayer cell cultures [18].

Materials and Methods

Tissue culture

Fibroblast cultures used were from frozen stock cultures of healthy donors. Cells were cultured as previously described [19]. Postmitotic stage in human fibroblasts was induced by irradiation of the cells (single dose of 7000 cGy using a Dermopan 2 instrument (Siemens, 50 kV, 1.0 mm Al filter, distance 5 cm) [20].

Rat brain astrocytoma cells (C6) and ROC-1 cells were subcultured and grown similarly to fibroblasts in Eagle's minimal essential medium containing 10% of fetal calf serum. 'ROC-1' cells are a hybrid clone between C6-rat astrocytoma cells and rat oligodendrocytes, kindly provided by Dr. F.A. McMorris (Wistar Institute, PA, U.S.A.), and were used in low subculture passages. Non transformed rat brain cells were grown in Dulbecco's modified minimal essential medium containing 10% of fetal calf serum. They were prepared and used as described for mouse brain cells [21].

Anisotropy determinations by fluorescence polarization

Cells were seeded in Petri dishes containing two glass coverslips (40 × 11 mm) at a density range of $12.5 \cdot 10^3$ to $250 \cdot 10^3$ cells/cm^2. Cultures were used not earlier than two days after trypsinization. Cells were prepared for polarization measurements by rinsing the coverslip cultures once in Hank's solution (37° C and pH 7.4).

The coverslips were placed in upright position into quartz glass cuvettes (Hellma SA, Switzerland) containing 5 μM TMA-DPH in 3 ml Hank's solution (37° C and pH 7.4). Special coverslip holders allowing only one geometrical configuration positioned the cell containing surface towards the incident light at an angle near 30°. This angle minimizes effects of selective reflection of the individual polarized components of the light [13,18].

Fluorescence anisotropy was determined with a Shimadzu RF-540 spectrofluorophotometer (L-format), equipped with a constant temperature four cell holder and two remote controlled rotating polarizers. This avoided opening the sample compartment during measurements. Fluorescence intensities were determined at four positions of the polarizers and fluorescence anisotropy (r_G) was calculated as follows:

$$r = \frac{I_{vv} - I_{vh}}{I_{vv} - 2I_{vh}}$$

$$G = \frac{I_{hv}}{I_{hh}}$$

$$r_G = \frac{I_{vv} - G_{vh}}{I_{vv} + 2G \cdot I_{vh}}$$

r, measured fluorescence anisotropy; G, correction factor for the optical system; r_G, corrected fluorescence anisotropy; $I_{vv(vh)}$, vertical excitation, vertical (horizontal) emission polarizer; $I_{hh(hv)}$, horizontal excitation, horizontal (vertical) emission polarizer. Excitation wavelength, 360 nm; emission wavelength, 430 nm. Slits: excitation, 5 nm: emission 20 nm.

Conditions and corrections of measurements

Labeling of the cultured cells by TMA-DPH was maximal after 5–10 min. Stable r_G values were read after 5 and up to 30 min. Nonlabeled cells at the excitation wave length of 360 nm and the emission wavelength of 430 nm gave total fluorescence intensities of less than 10 percent of the signals with TMA-DPH. Correction for autofluorescence of the cells and for the blanc of the dye in Hank's solution changed the fluorescence anisotropy values of the cell monolayers by less than 5 percent.

Materials and chemicals

Glass coverslips were obtained from Assistent (Altnau, Switzerland), minimal essential medium (MEM) with Earle's salt were purchased as dry powder from Seromed (Munich, F.R.G.). Fetal calf serum was from Boehringer (Mannheim, F.R.G.). Penicillin G was from Gist/Brokades (Delft, Holland). EGTA was purchased from Fluka (Buchs, Switzerland). TMA-DPH was obtained from Molecular Probes, Inc. (Eugene, OR, U.S.A.) and were kept as 2 mM ethanolic stock solution in the dark at 4° C. All other chemicals, solutions and solvents were of analytical grade and were purchased from Merck (Darmstadt, F.R.G.).

Results

Effects of culture conditions

Cell growth

Growing human skin fibroblasts, plated at low densities ($12.5 \cdot 10^3$/cm^2), showed a steady increase in fluorescence anisotropy values (r_G) with time in culture. The highest values were reached between day 6 and 11. r_G on day 11 was significantly ($P < 0.001$) higher than on day 2 (Fig. 1).

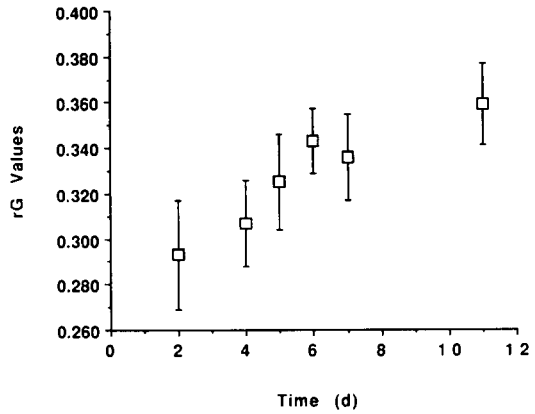

Fig. 1. Human skin fibroblasts were seeded at an initial cell density of $12.5 \cdot 10^3/cm^2$ and grown in Eagle's MEM containing 10% of fetal calf serum. Medium was changed on days 1, 4 and 7 after trypsinization. Fluorescence anisotropies were determined with 5 μM TMA-DPH in Hank's solution at 37 °C and pH 7.4 on days 2, 4, 5, 6, 7 and 11 after seeding. Data represent the means (±S.D.) of r_G of at least six independent coverslips.

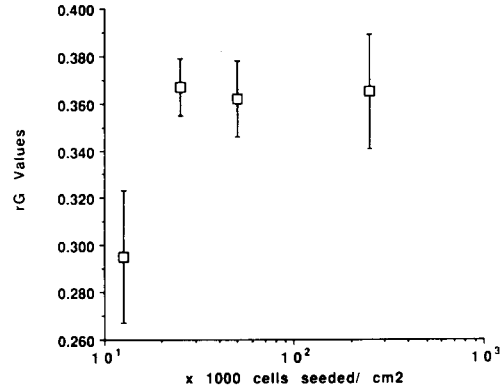

Fig. 3. Human skin fibroblasts were seeded at an initial cell density ranging from $12.5 \cdot 10^3$ to $250 \cdot 10^3/cm^2$ and were grown in Eagle's MEM containing 10% of fetal calf serum for 2 days. Anisotropy values were determined with 5 μM TMA-DPH in Hank's solution at 37 °C and pH 7.4. Data repreent the means (±S.D.) of r_G of at least six independent coverslips.

Feeding schedule

Fibroblasts, plated at $50 \cdot 10^3$ cells/cm^2 were fed on days 1, 4 and 7 after trypsinization (control condition). r_G values on day 10 were higher than values on day 6. Fluorescence anisotropy was similar in control and in daily fed cells. Starvation of fibroblasts by maintaining them in the same medium without change resulted in a significantly ($P < 0.01$) decreased anisotropy on day 10, but not on day 6 (Fig. 2). The starved cells were still alive by morphological criteria.

Cell density and growth restriction

Normal and X-ray irradiated fibroblasts were plated at increasing cell numbers and analyzed for anisotropy values 48 h after plating. r_G values in normal cells were independent of the seeding cell densities except at the lowest seeding density ($12.5 \cdot 10^3$ cells/cm^2). r_G values of irradiated (growth restricted) cells analyzed two days after plating at a density of $50 \cdot 10^3$ cells/cm^2 or higher revealed no differences when compared to nonirradiated cells of equal culture density (Fig. 3).

Effects of measurement conditions

Temperature

Confluent fibroblast monolayers were incubated in Hank's solution containing 5 μM TMA-DPH (pH 7.4) and r_G values were repeatedly determined at 37 °C during 25 min. Cells were then gradually cooled in the cuvettes to 4 °C and subsequently rewarmed to 37 °C. Temperature was controlled in a parallel cuvette by an electronic temperature sonde. Fluorescence anisotropy was determined at 37, 25, 20 and 4 °C in either direction of the temperature shift. Cooling of the cells from

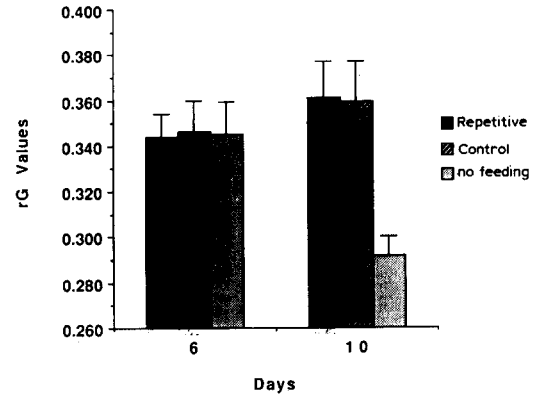

Fig. 2. Human skin fibroblasts were seeded at an initial cell density of $50 \cdot 10^3/cm^2$ and were grown in Eagle's MEM containing 10% of fetal calf serum. Cells were fed on days 1, 4 and 7 after trypsinization (control), daily (repetitive feeding) or medium was not changed after initial feeding. r_G-values were determined on days 4, 6 and 10 after trypsinization. Data represent the mean r_G values (±S.D.) of at least six independent coverslips.

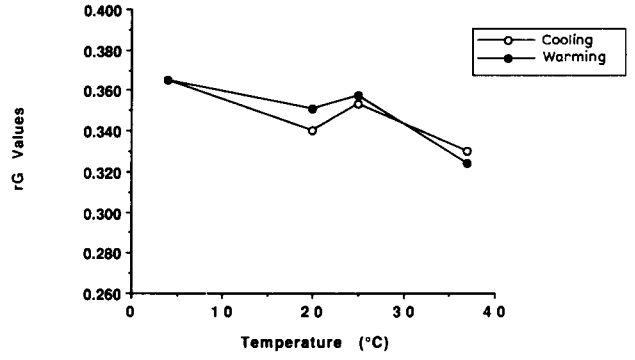

Fig. 4. Human skin fibroblasts were seeded at an initial cell density of $50 \cdot 10^3/cm^2$ and were grown in Eagle's MEM containing 10% of fetal calf serum for 2 days. Cultures on coverslips were incubated for 25 min in Hank's solution containing 5 μM TMA-DPH at 37 °C and pH 7.4, then gradually cooled to 4 °C and subsequently rewarmed. r_G values were determined at 37, 25, 20 and 4 °C in either direction. Data represent the mean r_G values of four independent experiments.

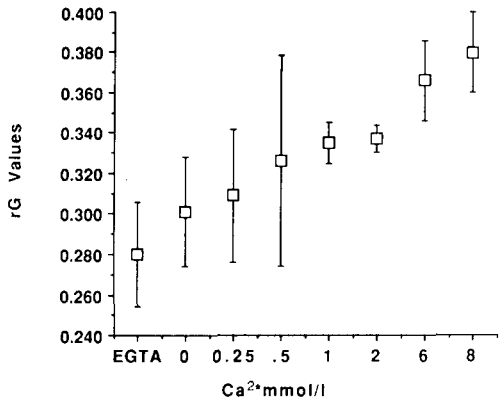

Fig. 5. Human skin fibroblasts were seeded at an initial cell density of $50 \cdot 10^3/cm^2$. Cells were grown in Eagle's MEM containing 10% of fetal calf serum for 2 days. r_G values were determined with 5 μM TMA-DPH in calcium free Hank's solution containing 1 mM EGTA or 0–8 mM Ca^{2+} at 37°C and pH 7.4. Data represent the mean r_G values (±S.D.) of at least six independent experiments.

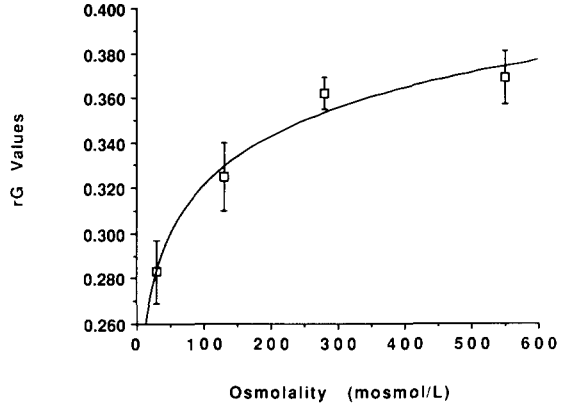

Fig. 6. Human skin fibroblasts were seeded at an initial cell density of $50 \cdot 10^3/cm^2$ and grown in Eagle's MEM containing 10% of fetal calf serum for 2 days. r_G values were determined with 5 μM TMA-DPH in aqueous solutions of mannitol (30–550 mosmol/kg H_2O) at 37°C. Data represent the mean r_G values (±S.D.) of at least six independent experiments.

37 to 4°C significantly increased r_G values with a reproducibled deviation from linearity in the range between 25 and 20°C. A similar relationship between temperature and r_G values was obtained during rewarming (Fig. 4).

Extracellular calcium

Anisotropy determinations in fibroblast monolayers were done in Hank's solution with Ca^{2+} concentrations ranging between 0 and 8 mmol/l and in Ca^{2+} free Hank's solution in presence of 1 mM EGTA. Anisotropy values were similar at 1 and 2 mM Ca^{2+}. At higher Ca^{2+} concentrations r_G values increased, at lower Ca^{2+} concentrations the values decreased but showed large variations. At 0 mM Ca^{2+} and 1 mM EGTA anisotropy was lowest (Fig. 5).

Incubation solutions of varying osmolalities

Fibroblast cultures were incubated in isotonic Hank's solution with 5 μM TMA-DPH for 20 min. Coverslips were then transferred either to fresh isotonic Hank's solution, to isotonic (280 mosmol/kg H_2O) solutions of saline or Hepes buffer (pH 7.4) or to hypotonic (30 mosmol/kg H_2O) solutions thereof as well as to 30–550 mosmol/kg H_2O mannitol, all containing 5 μM TMA-DPH. r_G values were repeatedly determined between 5 and 15 min. Transfer into fresh Hank's solution did not change the r_G values if compared to the preincubation values. In isotonic Hepes and mannitol solutions anisotropy slightly increased. Transfer into isotonic saline solution led to significantly higher r_G values ($P < 0.05$). In all the hypotonic solutions r_G values were significantly ($P < 0.01$) decreased by 15–20% (Table I).

A log-normal correlation of osmolality and r_G was observed for mannitol concentrations between 30 and 550 mosmol/kg H_2O (Fig. 6).

TABLE I

Fluorescence anisotropy r_G in varying isotonic and hypotonic solutions

Human skin fibroblasts were seeded at an initial cell density of $50 \cdot 10^3/cm^2$. Cells were grown in Eagle's MEM containing 10% of fetal calf serum for 2 days. Cells were incubated in Hank's solution for 20 min and subsequently transferred to various isotonic (280 mosmol/kg H_2O) and hypotonic (30 mosmol/kg H_2O) solutions, all containing 5 μM TMA-DPH. Data represent the mean r_G-values (±S.D.) of at least six independent experiments.

Solution	Osmolality	r_G
Hank's	280 mosmol/kg H_2O	0.348±0.017
NaCl 0.9%	280 mosmol/kg H_2O	0.370±0.021
Hepes buffer	280 mosmol/kg H_2O	0.356±0.011
Mannitol	280 mosmol/kg H_2O	0.362±0.007
NaCl	30 mosmol/kg H_2O	0.308±0.010
Hepes buffer	30 mosmol/kg H_2O	0.294±0.029
Mannitol	30 mosmol/kg H_2O	0.283±0.014

TABLE II

Fluorescence anisotropy r_G of human fibroblasts of different origins

Different types of fibroblasts and other cultured cells routinely used in our laboratory were seeded at a cell density of $12.5 \times 10^3/cm^2$ and grown to confluency in Eagle's MEM or in Dulbecco's modified MEM, all containing 10% of fetal calf serum, for 7 days. r_G-values were determined with 5 μM TMA-DPH in Hank's solution at 37°C and pH 7.4. Data represent the mean r_G-values (±S.D.) of at least six independent experiments.

Stratum papillare	0.350±0.015
Stratum reticulare	0.345±0.017
Urothelmyofibroblasts	0.352±0.006
Fluorescence anisotropy r_G of different rat cell types	
Astrocytoma cells	0.296±0.009
Brain cells	0.314±0.009
ROC-1 cells	0.313±0.016

Variability of fluorescence anisotropy among different cell strains

Similar anisotropy values were found in human fibroblasts of different origin when measured at comparable conditions (age, density). Significantly lower ($P < 0.05$) anisotropy values, however, were found in cultures of murine origin routinely used in our laboratory (rat brain astrocytoma cells (C6), non transformed rat brain cells and rat ROC-1 cells) (Table II). Aging of rat ROC-1 cells in culture (14 days) resulted in a further decrease in anisotropy values. Aging of astrocytoma cells in culture could not be followed since they detached from the coverslips after reaching high confluency. Non transformed rat brain cells increased in their anisotropy and reached maximal r_G values after 14 days in culture.

Discussion

Measurements of fluorescence anisotropy in living cells were performed in metabolically active membranes. The results were superior to those in membrane preparations, which do not maintain active ATP-generation and are usually contaminated by other cell organelles.

Most of the measurements in living cells have been performed in suspensions of scraped or trypsinized cultured cells. That includes a percentage of mechanically or chemically damaged cell membranes. Cultures on coverslips could be analyzed for membrane properties in situ. Damaged or dead cells that would be included within the cell suspensions were removed by rinsing the coverslip cultures prior to the measurement procedure. Furthermore there was neither sedimentation nor aggregation of the cells which occur in cell suspensions during the time of measurement. Thus coverslip preparations allowed to study also minor anisotropy changes. In cell suspensions such variations may additionally become masked by membrane fluidity artefacts produced during harvesting the cells. Measurements of fluorescence anisotropy in situ became specially useful when the results when compared with other membrane functions such as phagocytosis, pinocytosis or transport processes through membranes.

Determination of fluorescence anistropy is sensitive enough to measure membrane fluidity in situ while for other methods such as electron spin resonance (ESR) and magnetic resonance spectroscopy the ratio between signal and noise is usually too low in the limited cell numbers of a cell monolayer. The technique of determination of fluorescence anisotropy in situ was further improved by a special geometry of the coverslip culture within the cuvette reducing light scattering [13]. On the other hand the present method only allowed the use of few fluorescent dyes such as DPH or TMA-DPH while other fluorescent probes like *n*-(9-anthroxyloxy-) fatty acids are below the safe signal detection limit. TMA-DPH which was used in the study has the advantage that it remains in the superficial layers of the cell membrane and is hardly internalized into the cells during the measurement period [12]. TMA-DPH can be added in an ethanolic solution to the incubation medium. It is penetrating the membrane into a depth of 10 carbon atoms [11]. Furthermore, 5 μM TMA-DPH seemed to be non-toxic according to the fact that cells could be further cultivated for several days after the measurement (data not shown).

Our results demonstrate that fluorescence anisotropy increased in growing cultures after seeding at low density, while it remained constant in cultures seeded at near or complete confluency i.e. in cultures which were practically growth arrested. Effects of cell cycle and mitosis on membrane fluidity have previously been mentioned by De Laat et al. [14,15]. Growing cells were shown to have higher fluidity than $G_{1/0}$ arrested cells.

The feeding frequency had no major influence on fluorescence anisotropies of cultured human fibroblasts if the medium was exchanged daily or in intervals of 3–4 days. If, however, the medium of the cell cultures was not changed over a period of 10 days anisotropy values decreased between day 6 and 10 although cells appeared morphologically intact.

Anisotropy values of growth restricted cells which will terminally differentiate upon further culture were similar to the control values of non-irradiated cells. This was important in respect to functional integrity of cell membranes of irradiated and terminally differentiating cells and provided evidence that seeding above the $50 \cdot 10^3/cm^2$ was sufficient for standardization.

The composition of the media in which anisotropy measurements were performed were greatly influencing the results. An influence of extracellular calcium concentration on fluorescence anisotropy has been documented for hepatocyte plasma membranes in vitro [22] and for intestinal brush border and basolateral membranes [23]. To our knowledge, an extracellular calcium concentration dependent increase of anisotropy values has not yet been demonstrated in intact living cells. It is noteworthy that within the range of normal extracellular calcium concentrations the results of anisotropy values remained constant and also showed the smallest variations. The addition of EGTA to calcium free Hank's solution further reduced the anisotropy values. EDTA treatment as well as osmotic shock were shown to increase membrane fluidity of CHO cells grown on microcarriers [17].

Cell exposures to hypotonic solutions (30 mosmol/kg H_2O) greatly reduced anisotropy values possibly by osmotic stress whereas a hyperosmolar solution (550 mosmol mannitol/kg H_2O) had no comparable membrane effect.

Usually fluorescence anisotropies have been measured in Hank's solution at 37° C. As expected incubations temperature changed anisotropy values. The values were highest at 4° C and were gradually decreased as temperatures rised towards 37° C. Around room temperature (20–25° C) a temporary increase in anisotropy was observed. Interestingly enough this is the range of the transition temperature of some of the membrane lipids [24].

Fibroblast cultures from humans were explanted from different origin. In the body skin fibroblasts and urothel-myofibroblasts are exposed to different environmental temperatures. Growth temperature of culture cells does modify anisotropy and cells are partially able to adapt to changes of ambient temperature [25]. Differences in anisotropies may be present in the body but seem to be lost in cultured cells grown at the same temperature.

Transformed and non-transformed rat brain cells had similar anisotropy values but they were lower than those of human fibroblasts. We presently have no anisotropy values of rat fibroblasts, thus differences in membrane fluidity of the same cell type in varying species cannot be excluded. Interestingly prolonged incubation of rat brain cells resulted in an increase in fluorescence anisotropy while transformed cells that were partially contact inhibited (ROC-1 cells) were further lowering their anisotropy. This is consistent with findings by Shinitzky [6] on high membrane fluidity in malignancy.

In conclusion, TMA-DPH is a useful fluorescent probe for the determination of fluorescence anisotropies of substrate attached cell cultures. Cell growth and density must be standardized. Incubation conditions during the measurement such as temperature, calcium concentration and osmolality are modulators of fluorescence anisotropy.

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