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A PHOTOBLEACHING RECOVERY STUDY OF GLUCOCORTICOID EFFECTS ON LATERAL MOBILITIES OF A LIPID ANALOG IN S3G HeLa CELL MEMBRANES

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Treatment of the S3G strain of HeLa cells with dexamethasone inhibits cholesterol synthesis and thus results in decreased plasma membrane cholesterol-to-protein ratios. Incubation of HeLa cells with dexamethasone for 24 h lowers the steady-state fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) in intact cell plasma membranes and isolated plasma membranes (Johnston, D. and Melnykovych, G. (1980) Biochim. Biophys. Acta 596, 320–324). We have examined the effect of dexamethasone treatment of S3G HeLa cells on the lateral diffusion of the fluorescent lipid analogue 3,3′-dioctadecylindocarbocyanine iodide (DiI) by the fluorescence photobleaching recovery technique. The lateral diffusion of DiI was measured in cells 0, 2, 6, 12, and 24 h following treatment with dexamethasone and in cells identically handled without dexamethasone at 37°C. The diffusion constants of DiI in the treated and untreated cell membranes at zero time were $(4.52 \pm 0.30) \cdot 10^{-9}$ cm^2/s and $(4.56 \pm 0.24) \cdot 10^{-9}$ cm^2/s, respectively. There was no significant change in the lateral diffusion of DiI in the untreated cells over the 24 h period. The lateral diffusion of the lipid probe in the dexamethasone-treated cells began to increase 6 h following treatment and reached $(6.43 \pm 0.27) \cdot 10^{-9}$ cm^2/s at 24 h. The lateral diffusion of DiI was also measured at 25, 17, 10 and 4°C following 24 h incubation with and without dexamethasone. The effect of dexamethasone treatment on the lipid probe lateral diffusion observed at 37°C is decreased at 25°C and reversed in direction at 10 and 4°C. These results agree with those obtained in artificial systems containing varying amounts of cholesterol and support the suggestion that cholesterol acts to suppress phospholipid phase changes in animal cells. The lateral diffusion of DiI localized as a monolayer at a mineral oil-water interface was measured by fluorescence photobleaching recovery. The resulting data and the viscosity of the mineral oil were used to calculate the microviscosities of the plasma membranes of untreated and dexamethasone-treated cells at 25°C. Membrane microviscosities were also calculated from the fluorescence polarization studies cited above. In both cases the dexamethasone treatment reduced the apparent microviscosity by approximately 25%. However, the absolute microviscosity values obtained by the two techniques differ by a factor of 3.

Introduction

Various biological functions have been linked to the physical properties of eukaryotic cell plasma membranes. In particular, membrane permeability [1], immunogenicity [2], temperature adaptation [3,4], egg fertilization [5,6], and membrane-bound enzyme activity [7–9] have been related to the mobility of membrane lipids. Such correlations between so-called lipid fluidity and biological function remain speculative but stimulate interest in those physical factors that appear to control the

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Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene, DiI, 3,3′-dioctadecylindocarbocyanine iodide (diI-C_{18}-(3)).

lipid dynamics of biological membranes.

Experiments on the effect of cholesterol content on lipid mobility in biological membranes and artificial bilayers suggest that cholesterol increases the rigidity of the bilayer at high temperatures and increases the fluidity of the membrane at lower temperatures [10]. Several experimental approaches, including ESR spectroscopy [12,13], resonance energy transfer [14], excimer formation rates [15,16], fluorescence polarization [11,17–19], and fluorescence photobleaching recovery [20–23], have been utilized to determine the membrane fluidity of biological membranes and artificial lipid bilayers. Fluorescence polarization studies utilizing the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) in normal and leukemic lymphocytes led Shinitzky and Inbar [11,17] to propose their model for the regulatory function of cholesterol in the development of leukemia. Thompson and Axelrod [21] have utilized the recently-developed technique of fluorescence photobleaching recovery to examine the effect of cholesterol depletion of erythrocyte membranes on the lateral diffusion of the fluorescent lipid probe 3,3′-dioctadecylindocarbocyanine iodide (DiI). They reported that the lateral diffusion of the probe is 50% slower in the cholesterol-depleted membranes at −3°C but no difference in the probe diffusion is observed at 40°C. Searls and Edidin [23] have examined an embryonal cell line which exhibits slower lateral diffusion of DiI when compared to the ectodermal cells into which it differentiates upon treatment with retinoic acid. These differences are paralleled by markedly lower free cholesterol levels in the undifferentiated embryonal cells. Melnykovych and co-workers [24] demonstrated that treatment of HeLa cells with dexamethasone for 24 h inhibits cholesterol biosynthesis and decreases the cholesterol-to-protein ratio of the membrane from 19.1 to 16.4. Johnston and Melnykovych [25] reported that treatment with dexamethasone and 25-hydroxycholesterol, potent inhibitors of sterol biosynthesis, resulted in lower steady-state polarization values of DPH in intact cells, dispersions of whole cell lipid extracts, and isolated membrane fractions. We report here the effect of dexamethasone treatment on the lateral diffusion of DiI in membranes of the S3G strain of HeLa cells.

In view of previous work, there are three questions we attempted to answer by our measurements of lateral diffusion. (1) Is lipid diffusion in the membranes of HeLa cells affected by dexamethasone treatment? (2) How is such an effect expressed at different temperatures? (3) How do the results of fluorescence photobleaching recovery lateral mobility measurements compare with those previously obtained from fluorescence polarization studies in the same system?

Materials and Methods

Cell preparation

The origin of, and culture methods for, the S3G strain of HeLa cells have been published previously [9]. Cells used in the experiments reported here were incubated at 37°C for 24 h in minimal essential medium containing 2% fetal calf serum in 25 cm^2 petri dishes. Confluent cultures were selected and then incubated in minimal essential medium at 37°C with and without $2.8 \cdot 10^{-6}$ M dexamethasone. Representative cultures were removed at the selected times, labeled with DiI as described below and used immediately for the lateral diffusion measurements. Total cell cholesterol content was determined by gas-liquid chromatography [26]. The phospholipid analog DiI was synthesized according to the procedure of Sims et al. [27]. The DiI was dissolved in absolute ethanol to give a working solution of 2.5 mg/ml. The maximal absorption of DiI in this solvent is at 553 nm with an extinction coefficient of $1.2 \cdot 10^5$ $M^{-1} \cdot cm^{-1}$ and its fluorescence emission spectrum peaks at 565 nm. Cell cultures in 25 cm^2 petri dishes were washed twice with Hanks' balanced salts solution. Each culture was incubated at 2 ml of Hanks' balanced salts solution with 6 μg/ml DiI in 1% ethanol for 20 min at 37°C. Following incubation each culture was washed twice in 2 ml of Hanks' balanced salts solution to remove excess dye and used immediately for the fluorescence photobleaching recovery measurements.

Preparation of DiI monolayer

DiI dissolved in diethyl ether was layered on the surface of distilled deionized water in a 50 ml beaker. The diethyl ether was evaporated at 37°C for 1 h. A thin layer of U.S.P. extra heavy mineral oil (Denison Laboratories, Pawtucket, RI) was

carefully layered atop the surface of the water. The DiI monolayer was readily visualized at the water/oil interface using oil immersion optics while exciting DiI fluorescence with 514.5 nm laser illumination. Monolayers were prepared with 2 μg and 10 μg of DiI representing 5% and 25% coverage of the interfacial area, respectively. The lateral diffusion of DiI in each monolayer was measured at 22°C. The viscosity of the mineral oil was measured at selected temperatures in a calibrated Cannon-Fenske viscometer mounted in a water bath.

Fluorescence photobleaching recovery measurements

The fluorescence photobleaching recovery system used to measure diffusion constants is illustrated in Fig. 1. A description of the system has been published previously [28,29,30].

A × 40 oil immersion objective of Na 0.66 was used in the DiI monolayer photobleaching experiments. The objective used in the measurement of the lateral mobility of the DiI in the membranes of the HeLa cells is a X50 water immersion fluorescence objective of N.A. 1.00. The DiI emission is isolated using standard Zeiss filter and dichroic mirror sets for tetramethylrhodamine. Cell cultures are examined in the petri dishes in which they are grown on a thermoelectrically cooled/heated stage (Cambion, Cambridge, MA) with a temperature range of 0–60°C.

Data analysis

Our method of on-line processing of the fluorescence photobleaching recovery data has been previously published [28]. Machine language subroutines provide communication with the photon

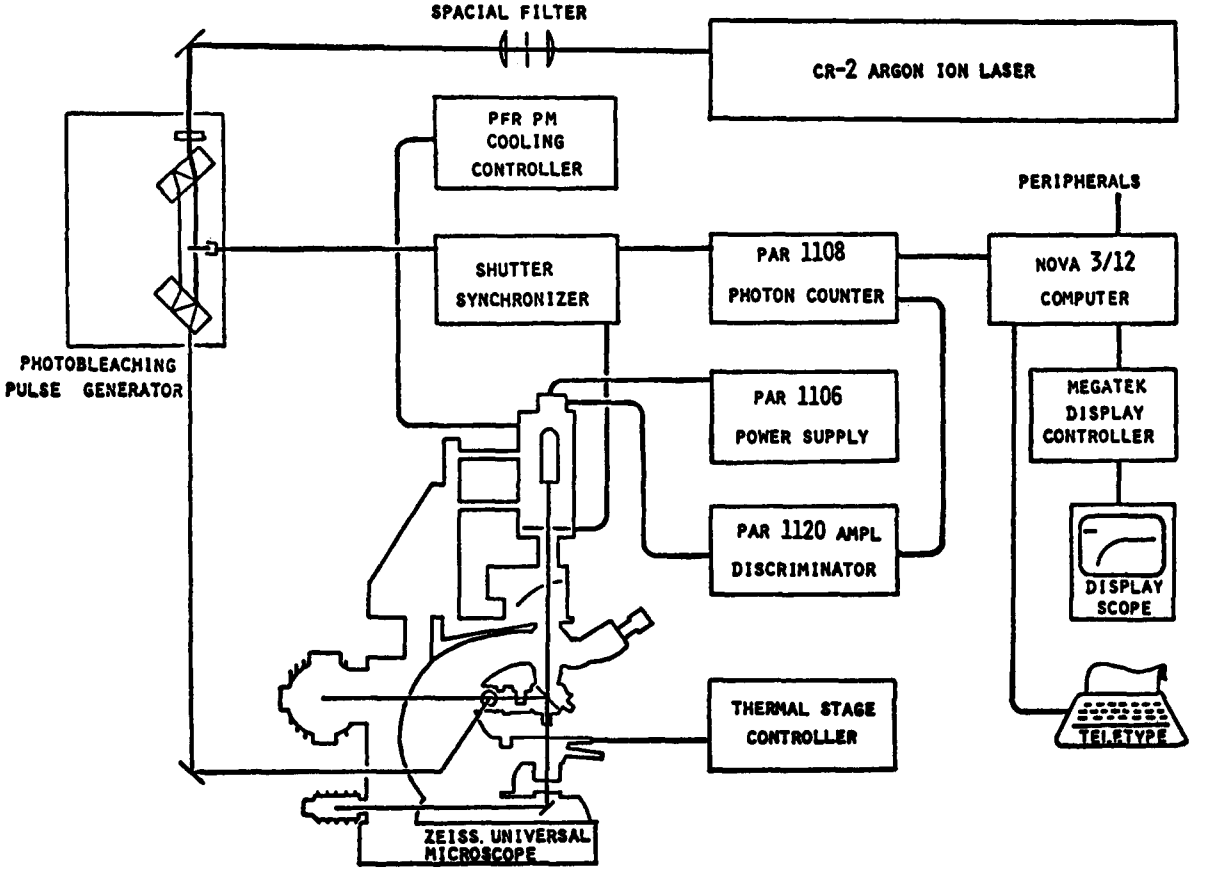

Fig. 1. Block diagram of fluorescence photobleaching recovery system used for cell surface studies. A description of the various components may be found in the text.

counter and the shutter synchronizer while the actual processing of the data is in BASIC. The half-time, $t_{1/2}$, of diffusive recovery following the bleaching pulse with a Gaussian beam profile is given by

$$t_{1/2} = \gamma r^2/4D \quad (1)$$

where γ is a factor dependent on the degree of photobleaching, r is the $1/e^2$ radius of the laser beam at the cell surface and d is the diffusion constant of the fluorescent species. The degree of photobleaching is measured by a parameter, K [31]. In our analysis we fit the raw data directly in terms of the parameters associated with a given measurement. The diffusion constants reported here represent an average value obtained from 10–45 cells. The diffusion constants measured on model systems are estimated to have an absolute accuracy of ±20%. The main source of uncertainty in model systems is the laser beam profile in the sample plane. The precision of such measurements is, however, closer to ±5%. The main difficulties in measuring phospholipid analog diffusion on HeLa cell surfaces are maintaining the beam focus on the upper plasma membrane and cell heterogeneity. We estimated that the diffusion constants of DiI in the plasma membrane of HeLa cells reported below are reproducible to ±15%.

The microviscosity of the cell membranes was evaluated by comparing DiI diffusion constants measured at mineral oil/water interfaces with diffusion constants measured on cell surfaces. The viscosity of the pure mineral oil used to form the interfaces was determined at selected temperatures as described earlier. The relationship of viscosity and diffusion may be expressed as

$$D \propto T/\eta \quad (2)$$

where D is the diffusion constant, T is the absolute temperature, and η is the viscosity of the medium in which the diffusion occurs. One knows all three parameters for a DiI monolayer at the mineral oil/water interface. This allows evaluation of the proportionality constant in Eqn. 2 and, hence, estimation of η for a cell membrane where D has been measured at a known temperature.

The method for evaluating membrane microviscosity from fluorescence polarization measurements has been previously published by Shinitzky and co-workers [17,32]. The foundation of the analysis is the Perrin equation

$$r_0/r = 1 + C(r)\frac{T\tau}{\bar{\eta}} \quad (3)$$

which describes the polarization of a fluorescent probe in a viscous medium. The measured and limiting fluorescence anisotropies are r and r_0, respectively; T is the absolute temperature, τ is the excited state lifetime of the probe, and $\bar{\eta}$ is the microviscosity of the medium which surrounds the DPH molecule. The final parameter $C(r)$ is related to the molecular shape of the fluorescent probe [17]. By means of a plot of r_0/r vs. $T\tau/\eta$ for DPH in purified liquid paraffin, $C(r)$ was estimated [33] at $(8.6 \pm 0.4) \cdot 10^5$ poise $\cdot$ deg$^{-1} \cdot$ s^{-1}. The values of r_0 and τ used in our data analysis were previously determined by Shinitzky and Inbar [17] as 0.362 and 11.4 ns, respectively.

Results

It has been previously demonstrated that acetate incorporation into the total nonsaponifiable lipids of HeLa cells under the conditions used in these experiments is an accurate measure of cholesterol synthesis [34,24,35]. Fig. 2 shows the effects of dexamethasone treatment on the incorporation of tritiated acetate in the nonsaponifiable lipid fractions of S3G HeLa cells [24]. A significant decrease in [^{3}H]acetate incorporation into the cholesterol fraction of the HeLa cells becomes apparent following 2–6 h of incubation in $1 \cdot 10^{-6}$ M dexamethasone. 24 h incubation in dexamethasone results in a decrease of radioactivity in the sterol fraction of about 50% as compared to untreated cells. This inhibition of cholesterol synthesis results in a cholesterol-to-protein ratio of 16.4 μg/mg in the dexamethasone-treated cells, which is significantly lower than the ratio of 19.1 μg/mg, observed in the untreated cells [25].

In order to assess the effects of dexamethasone treatment on the lipid mobility in the membrane, we measured the lateral mobility of DiI at 37°C following 0, 2, 6, 12 and 24 h incubation in $2.8 \cdot 10^{-6}$ M dexamethasone and in cells incubated under identical conditions without dexamethasone.

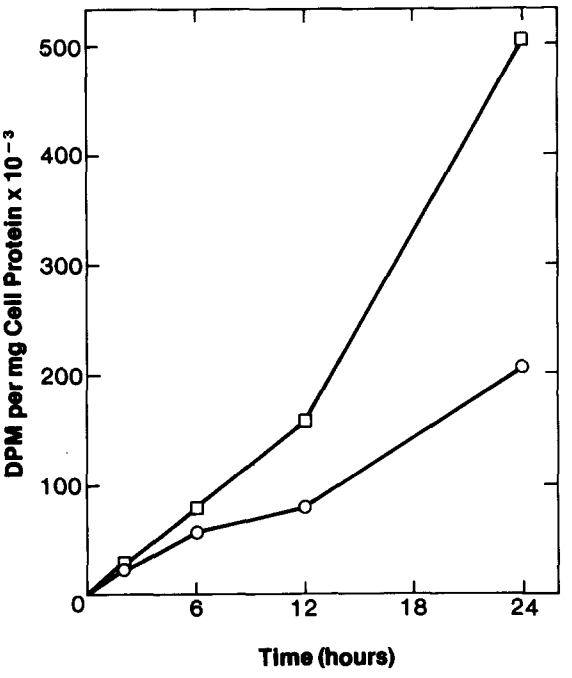

Fig. 2. Inhibition of [^{3}H]acetate incorporation into cholestrol fraction of HeLa cells. The cells were grown in 10% serum-supplemented Eagle's medium for 18 h and then changed to a serum-free medium that contained 1.0 μCi [^{3}H]acetate per ml. Incubation took place in either the presence (○) or the absence (□) of $2.8 \cdot 10^{-6}$ M dexamethasone. Individual points represent means of three separate cultures.

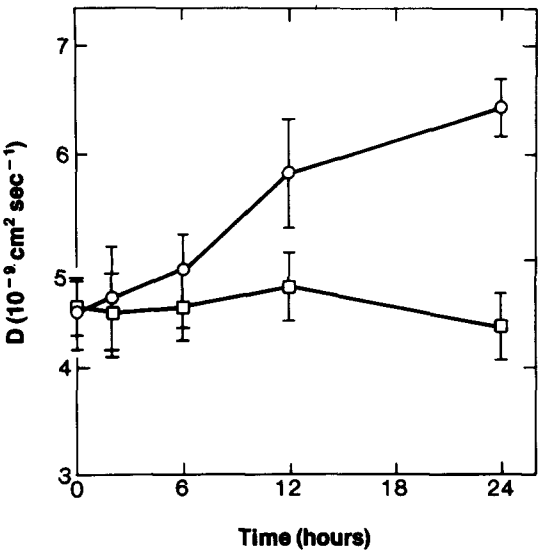

Fig. 3. Diffusion coefficients of DiI as a function of time following dexamethasone treatment. Cell cultures were incubated in minimal essential medium with (○) or without (□) $2.8 \cdot 10^{-6}$ M dexamethasone. At the specified times cell cultures were labeled with the same concentration of DiI. Each point represents an average of approximately 20 measurements on individual cells at 37°C.

Fig. 3 shows the results of this experiment. There is no significant change in the diffusion constant of DiI on untreated cells throughout the 24 h period of the experiment. Cells treated with dexamethasone begin to exhibit measurably increased lateral diffusion of DiI 6–12 h following treatment and a 50% increase in DiI diffusion after 24 h of incubation in dexamethasone. Comparison of Fig. 2 and Fig. 3 readily demonstrates the apparent correlation between increased DiI lateral diffusion and inhibition of cholesterol synthesis.

The inhibition of cholesterol synthesis resulted in increased lipid bilayer fluidity at 37°C as had been previously proposed [10,11]. We wondered if this effect reversed in direction when diffusion constants were measured at lower temperatures. Parallel sets of cell cultures incubated 24 h with and without dexamethasone were used to ascertain the effect of such treatment on the lipid mobility in the membranes at lower temperatures. The lateral diffusion of DiI in the membranes of these cells was measured at 25, 17, 10 and 4°C. The lateral diffusion constant of DiI in the dexamethasone-treated and untreated cells is shown as a function of temperature in Fig. 4. The lipid analog diffusion constant is more temperature-dependent in the dexamethasone-treated cells. Between 37°C and 4°C the diffusion constant of DiI in the membranes of the dexamethasone-treated cells decreases by a factor of 3.7 compared to a decrease of 1.5 in the untreated cells. At sufficiently low temperatures dexamethasone treatment decreases rates of probe diffusion: at 4°C DiI diffuses 40% more slowly in the treated cells than in untreated cells.

Johnston and Melnykovych [25] have previously published the results of steady-state fluorescence polarization studies of DPH on cells incubated 24 h with dexamethasone. Fluorescence polarizations of DPH have been used to calculate the microviscosity of cell membranes by comparison with DPH polarizations in homogeneous solvents of known viscosity [11, 17–19]. There are

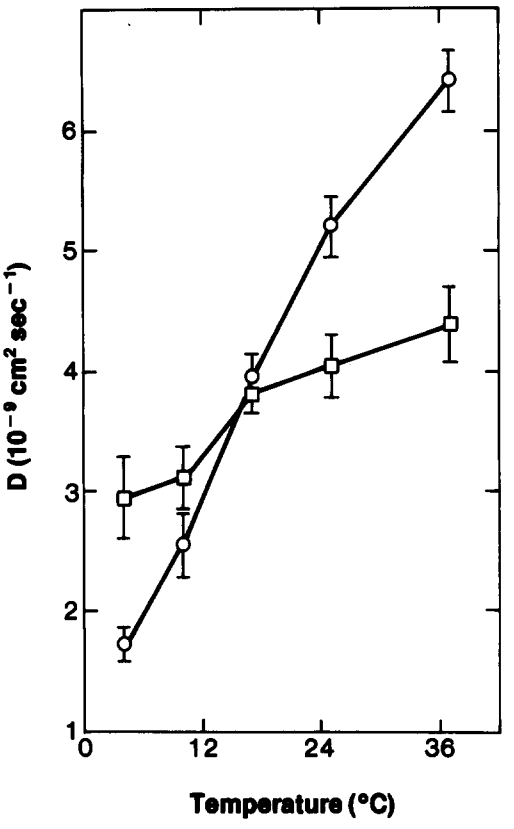

Fig. 4. Diffusion coefficients of DiI as a function of temperature following dexamethasone treatment. Cell cultures were incubated in minimal essential medium with (○) or without (□) $2.8 \cdot 10^{-6}$ M dexamethasone for 24 h. The cell cultures were labeled with the same concentration of DiI and the diffusion coefficients were measured at the specified temperatures. Each point is an average of approximately 20 measurements on individual cells.

good reasons to fear that quantitative membrane microviscosity estimated from polarizations of DPH fluorescence may not be meaningful. Such analyses of DPH fluorescence polarization have been complicated by recent studies showing that DPH is not confined to the sample membrane in intact cells [36], that DPH rotations measured in lipid vesicles exhibit hindered torsional motions [37], and that DPH fluorescence does not decay as a single exponential [38,39]. Our measurements of DPH polarizations and DiI diffusion constants in the dexamethasone-treated HeLa cells provide an excellent opportunity to assess the magnitude of this problem. We have calculated the membrane microviscosities of HeLa cells incubated with and without $1 \cdot 10^{-6}$ M dexamethasone from the DPH fluorescence polarization data previously published [25]. The results of these calculations are presented in Table I. To ascertain how these values compared with microviscosities calculated from direct fluorescence photobleaching recovery lateral diffusion measurements we devised a means of determining membrane microviscosities from the fluorescence photobleaching recovery data. The lateral diffusion of two concentrations of DiI localized as a monolayer at a mineral oil/water interface was measured by fluorescence photobleaching recovery. The resulting diffusion constant in a system of known viscosity was then used to calculate the membrane microviscosities from the fluorescence photobleaching recovery diffusion constants of dexamethasone-treated and untreated cells as outlined above. The results of these calculations are also presented in Table I. Both tech-

TABLE I

EFFECTS OF DEXAMETHASONE ON CHOLESTEROL CONTENT, STEADY-STATE FLUORESCENCE POLARIZATION, DiI DIFFUSION AND MEMBRANE MICROVISCOSITY IN S3G HeLa CELLS.

Cell growth and treatment of cells are described in Materials and Methods. Cholesterol/protein ratios are for three determinations of a single culture. The polarization and diffusion values represent the mean of multiple measurements. The experimental procedures are described in the text. All measurements were made at 25°C following 24 h incubation.

	Cholesterol/protein (μg/mg)	Polarization (P)	Viscosity from P (Poise)	Diffusion constant (D) (cm^2/s) ($\times 10^9$)	Viscosity from D (Poise)
Control	19 ±0.02	0.255±0.008	3.1	4.04±0.21	9.22
Dexamethasone	16.4± 1.5	0.227±0.010	2.07	5.21±0.26	7.15

niques indicate that membrane microviscosities are approx. 25% lower in the dexamethasone-treated cells. The absolute values of the viscosities calculated from the fluorescence photobleaching recovery data are larger by a factor of 3 than those calculated from the fluorescence polarization results.

Discussion

We have observed an increase in the lateral mobility of DiI in the membranes of dexamethasone-treated HeLa cells when examined at 37°C. This increase in DiI mobility correlates with the inhibition of cholesterol synthesis in HeLA cells identically treated with dexamethasone [24]. An analogous change in DiI diffusion has been observed by Searls and Edidin [23] to accompany increased membrane cholesterol resulting from differentiation of an embryo-derived cell line. The increased membrane fluidity with decreasing cholesterol content reflected by these mobility measurements has previously been suggested by studies of model membrane systems [10]. The lateral diffusion measurements of DiI in similarly treated cells at 4°C and 10°C show glucocorticoid effects of opposing magnitude. These results at low temperatures are consistent with Thompson and Axelrod's results on erythrocyte ghosts [21]. The low-temperature results are also in agreement with recent studies and analysis of model systems containing cholesterol [20,40–42]. It appears that the inhibition of cholesterol synthesis by dexamethasone and resulting lower cholesterol:protein ratios effect the fluidity of the membrane as reflected in the lateral mobility of DiI. Cholesterol appears to increase the ridigity of the HeLa cell membranes at high temperatures and increase the 'fluidity' at low temperatures.

Our lateral diffusion studies presented here also agree with previous experiments on the same system involving the rotational mobility of the fluorescent lipid probe DPH [25]. Although actual membrane microviscosities obtained by the two techniques are quite different and although DiI and DPH seek dissimilar membrane microenvironments, glucocorticoid effects are qualitatively similar in each case. The rotational mobility of DPH in membranes has been examined on the nanosecond time scale by time-resolved fluorescence anisotropy. Such studies have demonstrated that the fluorescence polarization of DPH decays to a non-zero stationary value within the fluorescence lifetime of the probe. Kinosita and coworkers [43] have published a theoretical model for such decay which considers the motion of the long axis of the molecule to be restricted to a cone the axis of which is normal to the plane of the membrane. The motion of the probe is then described by two parameters, a wobbling or rotational diffusion constant within the cone and the angle of the cone which represents the orientational constraint imposed on the probe by the neighboring membrane molecules. The consequences of such molecular orientation constraints to steady-state fluorescence polarization measurements involving DPH have been treated by Jähnig [44] and by Hildenbrand and Nicolau [45]. Both have concluded that steady-state fluorescence polarization data are best analyzed by using this 'static' component of steady-state polarization to evaluate an order parameter reflecting the degree of lipid orientation within the membrane. The utility of membrane microvicosities calculated from such data by the Perrin equation are, however, not discounted by these authors. Hildenbrand and Nicolau [45] point out that such parameters can provide generally correct qualitative information about changes in the ordering of membrane DPH in a response to various perturbations.

As in other systems studied [20,21,40] it seems that cholesterol suppresses temperature-dependent changes in the physical state of the HeLa cell membranes. It is clear that the temperature dependence of the lateral mobility of the DiI probe has been altered by steroid-induced membrane cholesterol depletion. It has been suggested [21], and we concur, that cholesterol may also decrease the dependence of diffusion upon the membrane phospholipid fatty acid composition. There is evidence in one biological system containing cholesterol that changes in fatty acid composition have minimal effect on DiI lateral mobility [46]. It is assumed that the apparent modulation of the phase transition seen in phospholipid bilayers following the addition of cholesterol also modulates the effect the fatty acid composition has on the physical dynamics of these bilayers and cholesterol-contain-

ing biomembranes. Consequently, while cholesterol acts to suppress temperature-induced changes in the dynamic state of plasma membranes, it appears that cholesterol may also serve to stabilize the physical properties of biological membranes against changes in phospholipid composition.

Glucocorticoids are known to modify the activities of certain membrane-bound enzymes such as alkaline phosphatase [8] and 5'-nucleotidase [9]. The question arises as to whether such effects can satisfactorily be explained by the current transcriptional control model for the glucocorticoid action [47]. This model involves binding of the steroid by specific cytoplasmic receptors and subsequent translocation of the receptor-steroid complex to the nucleus where interactions with chromatin modulate specific transcriptional events. Our results suggest that dexamethasone inhibition of cholesterol biosynthesis by such a mechanism and resulting changes in membrane composition can explain changes in the lateral mobility of membrane lipids which this steroid induces. It remains to be seen whether glucocorticoid effects on membrane-bound enzyme activities can similarly be explained by membrane compositional changes. For example, increases in alkaline phosphatase activity observed in HeLa cell membranes following treatment with microdispersions of lysophosphatidylcholine [8] might be understood in terms of compositional effects. Final resolution of this question must nevertheless await further experimental results.

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