The human promyelocytic leukemia cell line, HL-60, undergoes differentiation in the presence of dimethyl sulfoxide (DMSO), phorbol esters, and other agents. Studies were undertaken to determine whether agents that promote differentiation influence membrane fluidity and to assess whether changes in membrane fluidity occur during the process of differentiation. Cells were grown in the presence of DMSO, 1.25%, or tetradecanoyl-phorbol 13-acetate (TPA), 1.6 x 10^{-6} M. Surface membranes were isolated from cells by sucrose density sedimentation following cell disruption with nitrogen cavitation. Membrane fluidity was assessed by the fluorescence polarization of the membrane probe 1,6-diphenyl-1,3,5-hexatriene (DPH). A progressive decrease in membrane fluidity was first observed on day 3 following exposure of HL-60 cells to DMSO, and this reached a maximum on day 5, coincident with morphological and cytochemical differentiation. Similar changes in membrane fluidity were observed in liposomes prepared from extracted membrane lipids. The ratio of membrane cholesterol to phospholipid was increased 37% on day 5, and there was a small increase in the ratio of saturated to unsaturated fatty acids, with no significant change in phospholipid composition. No changes in membrane fluidity were observed in liposomes prepared from extracted membrane lipids. The ratio of membrane cholesterol to phospholipid was increased 37% on day 5, and there was a small increase in the ratio of saturated to unsaturated fatty acids, with no significant change in phospholipid composition. No changes in membrane fluidity were observed in liposomes prepared from extracted membrane lipids. The ratio of membrane cholesterol to phospholipid was increased 37% on day 5, and there was a small increase in the ratio of saturated to unsaturated fatty acids, with no significant change in phospholipid composition. No changes in membrane fluidity were observed in liposomes prepared from extracted membrane lipids.

**Plasma Membrane Isolation**

All isolation procedures were carried out at 4°C. HL-60 cells were harvested at logarithmic growth phase (~1 x 10^{6} cells/mL). Cells were centrifuged at 120 g (Sorvall RC-3 Centrifuge) and were washed twice with Hanks' balance salt solution (Grand Island Biological Company, Grand Island, N.Y.). The cells were resuspended in 0.14 M NaCl, 0.5 mM MgCl₂, and 2 mM HEPES pH 7.2 at a cell density of 2 x 10^{6} cells/mL. Equal volume of 0.5 M sucrose, 2 mM HEPES pH 7.5 was added dropwise to the cell suspension. Cells were then lysed by nitrogen cavitation in a pressure cell (Parr Instrument Co., Moline, Ill.) using 800 psi of nitrogen for 15 min. Na₂ EDTA was added immediately to the cell lysate to a final
concentration of 0.5 mM. Cell lysates were centrifuged at 5500 g for 15 min. The pellets were resuspended in the same volume of the lysis buffer and recentrifuged as before. The combined supernatant solutions were centrifuged at 100,000 g for 60 min. The pelleted crude membranes were resuspended in 25% sucrose solution with 2 mM HEPES, pH 7.2. The homogenized solution was overlaid on a 37% sucrose solution in a cellulose nitrate centrifuge tube. The sample was centrifuged for 90 min at 100,000 g in a Beckman S.W. 50.1 rotor (Beckman L-65 centrifuge). The membrane fraction at the interface was removed and resuspended with 1 mM HEPES at pH 7.2. The sample was then centrifuged for 90 min at 100,000 g. Pellets were resuspended in appropriate solutions.

`Biochemical Essays`

Protein concentration was determined by the method of Lowry et al. using bovine serum albumin as standard.\textsuperscript{10} (\(\text{Na}^+ + \text{K}^+\))-ATPase activities of plasma membranes and whole cell homogenates were measured by the method of Abdul and Kidweil et al.\textsuperscript{11} NADH-cytochrome-C reductase and 5'-nucleotidase were measured according to the method of Avruch et al.\textsuperscript{12} Succinate dehydrogenase was assayed by the procedure of DeDuve et al.\textsuperscript{13}

`Lipid Extraction and Analysis`

Extraction of lipids from aliquots of the aqueous plasma membrane suspension was performed with chloroform:methanol using the method of Bligh and Dyer.\textsuperscript{14} The chloroform:methanol-extracted lipids were assayed for cholesteryl\textsuperscript{15} and total lipid phosphorus.\textsuperscript{16}

Dispersions of membrane lipids were prepared by sonicating the dried chloroform:methanol lipid extracts at 30 W for 5 min under nitrogen in phosphate-buffered saline at 4°C using a Heat Systems (Long Island, N.Y.) model W375 sonifier.

`Fatty Acid Analysis`

All organic solvents used were analytical grade and were redis-tilled in glass. Fatty acid methyl esters of the extracted lipids were prepared according to Litman et al.\textsuperscript{17} Fatty acid analysis was performed by gas-liquid chromatography using a computerized Hewlett Packard 5830A gas chromatograph equipped with dual flame ionization detectors (Hewlett Packard, Avondale, Pa.) as described by Baron and Blough.\textsuperscript{18} A 6 ft x 2 mm. 10% Silar 10 C Gas Chrom Q 100/120 mesh column (Applied Science Lab. Inc., State College, Pa.) was used for quantitation of fatty acids. The temperature was held at 155°C for 0.5 min after injection of sample, rising to 190°C at 1°C/min, and held at 190°C for 10 min. Nitrogen flow was 25 ml/min. Identification of the various peaks was made by comparison with known standards.

`Phospholipid Analysis`

Phospholipid mixtures extracted from plasma membrane by chloroform:methanol were separated by thin-layer chromatography on Whatman 200-\(\mu\) thick 10 x 10 cm LHP-K preabsorbent plates (Whatman Inc., Clifton, N.J.). Approximately 5 \(\mu\)g of phospholipids was applied to the plate. The developing mobile phase was a mixture of chloroform:methanol:acetic-acid:water in the ratio of 25:15:4:2. After developing, the plate was dried for 5 min at 60°C before being sprayed with reagents. The spray reagent was made with cupric acetate, 30 g/liter, in 8% phosphoric acid.\textsuperscript{19} The plate was then dried for 20 min at 130°C. Phospholipid spots were analyzed by using a Schoeffel dual-beam spectrodensitometer (model 3000) equipped with a density computer (Schoeffel Instrument Corp., Westwood, N.J.) according to Touchstone et al.\textsuperscript{20}

`Fluorescence Polarization Measurements`

Measurement of fluorescence polarization were performed with an Elscent MV-1 microviscosimeter (Elscent Corp., Hackensack, N.J.) as reported previously.\textsuperscript{21,22} The fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene (DPH) (Aldrich Chemical Co., Milwaukee, Wisc.) was dissolved in tetrahydrofuran at 10\(^{-3}\) M and diluted 1000-fold with phosphate-buffered saline with vigorous mixing immediately prior to use. One volume of the diluted DPH dispersion was added to 1 volume of plasma membrane preparation at a protein concentration of 250 \(\mu\)g/ml and the mixture was incubated at 37°C for 30 min. Samples were diluted to optical density of less than 0.1 for measurements of fluorescence polarization. Light scattering itself did not cause depolarization of fluorescence, as monitored by means of serial dilutions of samples. Fluorescence polarization (\(P\)) was calculated from the intensity of emitted light parallel and perpendicular to the plane of excitation according to the formula.

\[
P = \frac{I_1 - I_0}{I_1 + I_0}
\]

\textbf{RESULTS}

`Membrane Purification`

Enzyme markers were used to assess the purity of the plasma membrane preparation. 5'-Nucleotidase activity, a commonly used plasma membrane enzyme marker, was not detectable either in isolated cell membranes or whole cell homogenates. However, (\(\text{Na}^+ - \text{K}^+\))-ATPase activity was present. As compared with the activity in the whole cell homogenate, there was a 15-fold enhancement of (\(\text{Na}^+ - \text{K}^+\))-ATPase activity in 6 separate plasma membrane preparations isolated from either DMSO-induced HL-60 cells or from HL-60 cells growing in continuous culture (Table 1). The activity of NADH-cytochrome-C reductase, an enzyme present in endoplasmic reticulum, was increased by an average of 1.4-fold in the plasma membrane fraction. No mitochondrial succinic dehydrogenase activity was detectable in the purified membrane fraction.

`Membrane Fluidity Changes During Cellular Differentiation`

The maturation of HL-60 cells is maximal 5–7 days after the addition of 1.25% DMSO.\textsuperscript{6} Membrane fluidity was measured in HL-60 cells during this period of differentiation using the hydrophobic fluorescent probe, DPH (Fig. 1). No observable differences were seen during the first 2 days after the addition of DMSO. Thereafter, the fluorescence polarization of DPH increased, indicative of a decrease in membrane fluidity. The maximum change in fluidity was observed at 5 days, when over 90% of the cells were differentiated as judged by the presence of myelocytes, metamyelocytes, and bands on Wright-stained smears. Thus, plasma membrane fluidity changes paralleled the morphological appearance of differentiated cells. The membrane fluidity of HL-60 cells was also
analyzed after exposure of the cells to $1.6 \times 10^{-8} M$ TPA, a phorbol ester. Because TPA induces cells to adhere to the plastic substrate after approximately 18 hr of exposure, studies of membrane fluidity were carried out for only the first 18 hr. Membrane fluidity at 18 hr was identical in HL-60 cells, whether or not they had been exposed to TPA (Fig. 1).

Figure 2 shows the fluorescence polarization of DPH in the plasma membrane fraction of HL-60 cells 5 days after exposure to DMSO. Membranes isolated from differentiated cells were less fluid than the membranes from HL-60 cells in continuous culture, as indicated by the significantly higher values for fluorescence polarization in the induced cells at all temperatures. As expected, no phase transition was observed over the temperature range of $10^\circ$-$45^\circ$. Since DMSO elutes from cells during the process of membrane isolation, measurements of fluorescence polarization were also made in the presence of 1.25% DMSO. No effect was observed when this concentration of DMSO was added to isolated membranes. Similarly, 5.0% DMSO had no effect on the fluidity of red cell ghost membranes.

**Analysis of Membrane Lipids**

To determine whether the observed changes in membrane fluidity were due to changes in membrane lipids, the fluorescence polarization of DPH was measured in liposomes prepared from lipids that had been extracted from the isolated membrane fractions. As seen with whole membranes, the membrane lipids of HL-60 cells 5 days after induction with DMSO were less fluid than the lipids extracted from HL-60 cells in continuous culture (Fig. 3).

In various biologic systems, membrane fluidity has been found to be influenced by the relative amounts of cholesterol and phospholipid, by the specific classes of phospholipids, and by the degree of saturation of the fatty acids present in phospholipids. These three characteristics of lipids were analyzed quantitatively in membranes from HL-60 cells in continuous culture and from HL-60 cells 5 days after exposure to DMSO.

There was no statistically significant difference in the relative quantities of the various phospholipids present before and following differentiation (Table 2). An analysis of the fatty acyl chains present in the membrane lipid extract is shown in Table 3. Compared with HL-60 cells in continuous culture, DMSO-differentiated HL-60 cells had slightly more saturated fatty acids with a small decrease in 16:1 and 18:1. The cholesterol/phospholipid (C/P) mole ratio of the plasma membrane fraction increased from 0.35 in continuously growing HL-60 cells to 0.48 after

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**Table 1. Enzymatic Activity of Whole HL-60 Cell Homogenates and of Membrane Fractions of HL-60 Cells**

<table>
<thead>
<tr>
<th></th>
<th>Whole Cell Homogenate</th>
<th>Membrane Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Growing Cells</td>
<td>DMSO-Induced Cells</td>
</tr>
<tr>
<td>(Na⁺-K⁻)-ATPase OD at 700 nM/mg protein/hr</td>
<td>2.14 (1.82-2.40)*</td>
<td>32.1 (25.2-40.1)</td>
</tr>
<tr>
<td>Cytochrome-C reductase</td>
<td>3.8</td>
<td>5.6 (5.5-5.7)</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>0.28</td>
<td>0</td>
</tr>
<tr>
<td>µmole/mg protein/hr</td>
<td>0.25</td>
<td>0</td>
</tr>
</tbody>
</table>

*Range.
Individual experiment. DMSO-induced cells were studied on day 5. A 37% increase in C/P was associated with a change in fluorescence polarization of 0.020. When expressed in terms of microviscosity, as in previous publications from our laboratory and from Shinitsky and coworkers, this represents a change of 0.59 poise (from 2.73 poise before differentiation to 3.33 poise at day 5). In our previous studies with red cells, a 37% increase in membrane cholesterol above baseline values resulted in an increase in microviscosity of 0.60 poise, and a 37% increase in platelet cholesterol led to an increase in microviscosity of 0.52 poise. Thus, the change in C/P in HL-60 cells on differentiation was of a magnitude that could account for the observed changes in fluidity observed with DPH.

DISCUSSION

These studies demonstrate that myeloid differentiation is associated with a progressive decrease in membrane fluidity and that this is the result of an increase in the cholesterol/phospholipid mole ratio and in the degree of fatty acyl chain saturation within the cell membrane. These observations are consistent with data reported by Gottfried showing that the C/P mole ratio of myeloblasts in acute leukemia is lower than that of mature polymorphonuclear leukocytes. Recently, Klock and Pieprzyk demonstrated that normal immature bone marrow cells also have a low C/P, suggesting that a low C/P is a manifestation of immaturity rather than of malignancy. Like HL-60 cells, Friend erythroleukemia cell membranes also undergo a decrease in membrane fluidity during DMSO-induced differentiation. Similarly, the membrane fluidity of chicken embryo fibroblasts decreases due to an increased C/P when these cells become confluent in vitro. It is likely that changes in membrane lipid composition and membrane fluidity modify the enzymatic and transport properties of cells as they assume their new physiologic role.

Do inducers of cellular differentiation evoke their signal through primary changes in membrane fluidity? Previous studies have shown that high concentrations of DMSO increase the phase transition temperature of phospholipid vesicles, suggesting that DMSO decreases membrane fluidity. We have confirmed these studies in our laboratory but have noted that, at the concentrations of DMSO utilized to induce differentiation, these changes in vesicles are not measurable (Ip and Cooper, unpublished). Moreover, no change in HL-60 membrane fluidity was observed using these concentrations of DMSO. Phorbol esters have also been reported to effect membrane fluidity. For example, TPA has been noted to increase the membrane fluidity of rat embryo cells and of a variety of

Table 2. Plasma Membrane Phospholipid Composition of DMSO-Induced and Continuously Growing HL-60 Promyelocytic Leukemia Cells

<table>
<thead>
<tr>
<th>Percent Total Phospholipids</th>
<th>HL-60 DMSO-Induced</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidyl ethanolamine</td>
<td>37.6 ± 1.4</td>
</tr>
<tr>
<td>Phosphatidyl serine + phosphatidyl inositol</td>
<td>19.1 ± 0.5</td>
</tr>
<tr>
<td>Phosphatidyl choline</td>
<td>33.0 ± 1.1</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>10.3 ± 0.5</td>
</tr>
<tr>
<td>Cholesterol/phospholipid</td>
<td>0.35</td>
</tr>
</tbody>
</table>

Results are an average of three separate pairs of experiments ± standard deviation. Triplicate TLC measurements were made for each individual experiment. DMSO-induced cells were studied on day 5.

Table 3. Fatty Acyl Chains of Plasma Membrane Lipids

<table>
<thead>
<tr>
<th>Acyl Group</th>
<th>Percent of Total Fatty Acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL-60</td>
<td>DMSO-Induced HL-60</td>
</tr>
<tr>
<td>14:0</td>
<td>3.9</td>
</tr>
<tr>
<td>16:0</td>
<td>26.8</td>
</tr>
<tr>
<td>16:1</td>
<td>5.1</td>
</tr>
<tr>
<td>18:0</td>
<td>22.4</td>
</tr>
<tr>
<td>18:1</td>
<td>15.4</td>
</tr>
<tr>
<td>18:2</td>
<td>2.3</td>
</tr>
<tr>
<td>20:1</td>
<td>0.7</td>
</tr>
<tr>
<td>20:2 + 22:0</td>
<td>0.7</td>
</tr>
<tr>
<td>20:3 + 20:4</td>
<td>5.9</td>
</tr>
<tr>
<td>20:5</td>
<td>1.8</td>
</tr>
<tr>
<td>22:1</td>
<td>2.4</td>
</tr>
<tr>
<td>22:5 + 24:1</td>
<td>2.5</td>
</tr>
<tr>
<td>22:6</td>
<td>3.2</td>
</tr>
<tr>
<td>24:0</td>
<td>1.5</td>
</tr>
<tr>
<td>Unsaturated/ saturated</td>
<td>41.9</td>
</tr>
</tbody>
</table>

Results are an average of two separate experiments. DMSO-induced cells were studied on day 5.
MEMBRANE FLUIDITY IN HL-60 CELLS

lymphoid cells at concentrations that are at or just above the range utilized to induce HL-60 cell differentiation. In the case of lymphoid cells, changes were noted only in those cell lines that became adherent and, among those cell lines, only in the adherent subpopulation. This suggests that the changes in fluidity that were observed may have been a manifestation of the TPA effect on cells rather than an early signal leading to that effect. In the present study we were unable to detect any early change in the membrane fluidity of HL-60 cells utilizing the same probe of membrane structure that was utilized in these previous studies.

It should be emphasized that measurements of membrane fluidity with probes such as DPH report average values for the bulk fluidity of the entire membrane, and local changes in fluidity that might be of great functional importance may not be detected. Thus, although such local changes may exist, it does not appear that either DMSO or TPA induces generalized effects on membrane fluidity in HL-60 cells at concentrations that induce differentiation. Rather, the changes in fluidity we have observed appear to characterize the process of cellular differentiation.

ACKNOWLEDGMENT

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Decreased membrane fluidity during differentiation of human promyelocytic leukemia cells in culture

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