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Fatty Acid Chain Flexibility in the Membranes of Normal and Transformed Fibroblasts

(membrane fluidity/transformed cell/3T3 cells/spin labeling)

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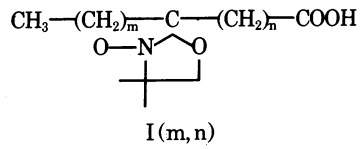
ABSTRACT **Fatty acid spin labels were used to measure the inherent flexibility of the lipid acyl chains in intact cell membranes of 3T3 mouse fibroblasts and several varieties of transformed 3T3 cells. No significant differences in inherent lipid flexibility were detected in the normal and transformed mouse fibroblasts. These results are compared with similar results obtained earlier for chick embryo fibroblasts. For the cells studied, a difference in the mobility of membrane glycoproteins of normal and transformed fibroblasts has either been demonstrated directly or inferred from differential agglutinability by other investigations. Therefore, a basis for correlating glycoprotein mobility and lipid motion in these cells was not found.**

The "fluidity" of membrane lipids has often been suggested as a possible factor that contributes to the differences in mobility of lectin-binding glycoproteins on the surfaces of normal and transformed cells. Evidence for differential protein mobility comes largely from experiments with plant lectins, which bind to cell surface glycoproteins and cause transformed cells to agglutinate more readily than normal cells (1). For instance, although the inherent distribution of binding sites is dispersed on the surfaces of both normal and transformed fibroblasts (2), lectins are able to induce the formation of clusters of binding sites, under certain conditions, only on the transformed cells (2, 3). There are several ways that the mobility and distribution of membrane glycoproteins, or proteins, may be related to the physical properties of membrane lipids. (*a*) The motions of membrane lipids may contribute to an "apparent microviscosity" for motions of those proteins that are free to rotate or diffuse in fluid lipid regions. In this case, the motions of the lipids may be influenced by fatty acid composition (4) and ratios of cholesterol to phospholipid (5) or by the presence of the membrane proteins themselves (6–8). (*b*) Lateral phase separations in membranes may include protein as well as lipid components (9). (*c*) Interactions of membrane proteins or receptors with such elements as microfilaments or microtubules may result in secondary effects in the membrane which are detectable as small changes in motions of membrane lipids (10, 11). The present paper is limited to (*a*) above: specifically, to the question of whether normal and transformed cells for which differential mobility of lectin-binding proteins has been demonstrated, or inferred from differential agglutinability, also differ measurably in the motions of membrane lipids.

Lipid motion, in terms of flexibility of lipid chains (12), is measured in this report by paramagnetic spin labels (13). Spin label measurements were made in intact cells of normal and several varieties of transformed 3T3 mouse fibroblasts. The present results with mouse fibroblasts are compared with earlier spin label measurements in normal and Rous sarcoma virus (RSV)-transformed chick embryo fibroblasts (14, 15). Both transformed 3T3 cells and RSV-transformed chick fibroblasts have been shown to agglutinate more readily than the normal counterpart (1, 16–18). Further, the ability of lectins to induce clustered arrangements of receptor sites on transformed mouse fibroblasts has been repeatedly demonstrated (1–3). Differences in fatty acid composition (14, 16, 19) are small for both normal and transformed mouse fibroblasts and for normal and RSV-transformed chick embryo fibroblasts, and cholesterol/phospholipid ratios are similar in normal and transformed chick fibroblasts (20, 21). However, the flexibility of lipid chains is not always determined by chemical composition alone, but may also be affected by the presence of proteins in the intact membrane. As an example, the motions of the lipids in Sindbis virus are dominated by the presence of the viral proteins (8). Thus, the question of whether differences in glycoprotein mobility may be correlated with measurable differences in lipid motion can best be answered by measurements of lipid motion in the intact cell membranes.

The spin-labeled fatty acids, I(m,n), provide a measure of



I(m,n)

the amplitude of motion of the molecular long axis about the average orientation of the fatty acid chain in the lipid bilayer. The amplitude of motion of these labels is sensitive to the flexibility of membrane lipid chains. Among the advantages of using labels I(m,n) to probe the motions of membrane lipids are the extensive theoretical analyses of the paramagnetic resonance spectra (12, 22) and the fact that measurements with these labels may be made quickly and with fairly low concentrations of label.

A published report (23) of detection of relatively large differences in lipid flexibility in normal and transformed mouse fibroblasts is in marked contrast with the results given here.

MATERIALS AND METHODS

3T3 Cells. Normal 3T3 and A_{31}, simian virus 40-transformed SVT_2, transformed Py_6, and revertant Py_6R_1 cells were a

Abbreviation: RSV, Rous sarcoma virus.

gift of Dr. T. Benjamin, Harvard Medical School. The cells were grown to confluence on 100-mm petri dishes in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated calf serum (GIBCO) for normal cells and 5% calf serum for transformed cells.

Spin Labeling Cells. The fatty acid spin labels I(m,n) were synthesized by the method described for (m,n) = (10,3), (10,4), (7,6), (5,10), and (1,14) (22). For label I(10,4): Analysis calcd. for $C_{21}H_{40}NO_4$: C, 68.07, H, 10.88; N, 3.78. Found: C, 67.96; H, 10.68; N, 3.71.

Procedure A. Cells attached to the dish were washed three times with calcium, magnesium-free phosphate-buffered saline (pH 7.3) at 37° and scraped from the dish with a rubber policeman. Cells were dispersed in 1 ml of phosphate-buffered saline containing 25–50 μM spin label, pelleted by low-speed centrifugation, washed once with label-free phosphate-buffered saline, pelleted, and used immediately for spectral measurements.

Procedure B. Cells on 100-mm dishes were washed with serum-free medium, covered with 5 ml of serum-free medium containing 50 μM spin label, and incubated for 30 min at 37°. The cells were washed and pelleted as in procedure A.

Paramagnetic Resonance Spectra. Paramagnetic resonance signals of labeled cells were recorded on a Varian E-9 spectrometer equipped with a variable temperature controller. The recorder scan width was calibrated with Fremy's salt (24) and found to be accurate to ±0.1 G. The samples consisted of about 6×10^6 to 1×10^7 cells contained in 50-μl glass disposable pipettes (Corning) sealed at one end. Spectra were recorded within 10–45 min after introduction of the spin label. When the spectra of several samples were recorded repeatedly over 1 hr, no changes in the order parameter were observed. Also, no differences in order parameter were observed when cells were labeled by either of the procedures (A or B) above. During a 1-hr period, there was considerable reduction (half-time <30 min) in amplitude of the signal, presumably due to chemical reduction of the paramagnetic nitroxide group by cell components. Modulation amplitudes were 1 G or less, and the microwave power was kept at or below 10 mW to avoid sample heating and signal saturation. The usual scan times were 8–16 min, and the time constants were 0.3 to 1.0. The temperature was monitored by using a copper-constantin thermocouple placed just above the resonance area of the cavity. For a single set of measurements comparing normal and transformed cells, the settings on the temperature controller and the position of the sample holder were not varied.

RESULTS

Factors That Affect the Order Parameters of Fatty Acid Spin Labels in Lipid Bilayers. Analysis of the paramagnetic resonance spectra of fatty acid spin labels in phospholipid bilayers has been discussed (12, 22). Briefly, certain spectral parameters are used to calculate order parameters (25). Order parameters are related to the mean angular deviation of the labeled fatty acid chain from its average orientation in the bilayer. High values of order parameters are characteristic of relatively solid lipid, and low values characterize very mobile lipids. In addition, in a given phospholipid bilayer, there is a gradient in order parameter as the label position is varied on the fatty acid chain. Characteristically, order parameters are high near the polar head groups and low in the center of the bilayer near the terminal methyl ends of the chains.

TABLE 1. *Order parameters* of fatty acid spin labels in mixtures of synthetic lecithins*

A: Measurements with label I(10,3) in dioleoyl (DOL) and diarachidonoyl (DAL) lecithin mixtures

DOL:DAL ratio	Order parameter* at: 37°
1:0	0.553
9:1	0.541
4:1	0.532
1:1	0.518
0:1	0.497

B. Measurements with label I(5,10) in dipalmitoyl (DPL) and dioleoyl (DOL) lecithin mixtures

DPL:DOL ratio	Order parameter† at 36°	41.5°
1:0	0.455	0.254
3:1	0.295	0.237
1:1	0.264	0.220
0:1	0.241	0.207

* The experimental values used to calculate order parameters are $T_\parallel'$ and $T_\perp'$ (in gauss). $T_\parallel'$ and $T_\perp'$ are equal to $^1/_2$ the separations of the outer and inner spectral extrema, respectively. The observed value of $T_\parallel'$ differs from the real value by a factor related to $(T_\parallel' - T_\perp')$. Order parameters, taking into account the correction to $T_\perp'$, are calculated according to the following equations (25):

$$\text{order parameter} = S = \frac{T_\parallel' - T_\perp' - C}{T_\parallel' - 2T_\perp' + 2C} \times 1.723$$

where $$C = 1.4 \text{ G} - 0.053 (T_\parallel' - T_\perp').$$

† In spectra for label I(5,10), only the inner extrema were well resolved. Order parameter calculations were similar to those described above except that $(T_\parallel' + 2T_\perp')$ was taken as 44.5 G in all cases; $T_\parallel'$ was calculated from $T_\parallel' = 44.5 - 2T_\perp'$; the correction to the observed value of $T_\perp'$ was taken as 0.8 G in each case.

The factors that have been demonstrated to affect the order parameter of the fatty acid spin labels in membranes include lipid composition (4, 5), the presence of some membrane proteins (6–8), anesthetics (26, 27), and interaction of membranes and membrane receptors with certain drugs and small molecules (10, 11). Table 1 demonstrates the sensitivity of two of the labels to fatty acid composition of lipid bilayers. In the data of part A of Table 1 and in the measurements done at 41.5° in part B, the order parameters vary as the lipid composition is varied. In each of these cases, the more unsaturated lipid of the pair (diarachidonoyl lecithin in part A and dioleoyl lecithin in part B) has a higher inherent amplitude of motion (lower value of order parameter). In mixtures of two lipid components, increasing amounts of the more saturated component (dioleoyl lecithin in part A and dipalmitoyl lecithin in part B) result in decreases in motion and higher order parameters. These measurements are made with labels that have the spin label portion located at two different positions on the fatty acid chain. From the data of Table 1, part A and part B at 41.5°, it appears that the motions of fatty acid spin labels are sensitive to differences of about 10% in the number of unsaturated fatty acid chains in a fluid

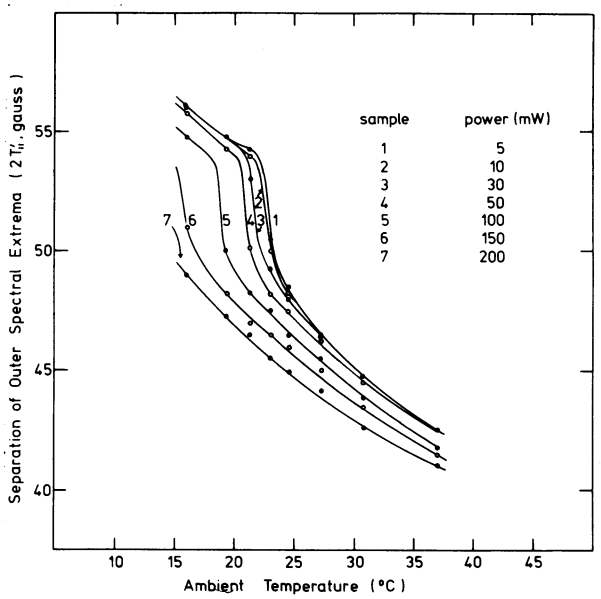


FIG. 1. Measurement of the displacement of sample temperature from the ambient temperature of the electron paramagnetic resonance cavity was made by using liposomes of dimyristoyl lecithin and a phospholipid spin label [prepared from egg yolk lysolecithin and I(7,6)] as an internal standard for temperature. Approximately 10^7 cells (chick embryo fibroblasts), mixed with 2 ml of a 2% suspension of spin-labeled dimyristoyl lecithin liposomes, were pelleted by low-speed centrifugation for spectral measurements. Curves 1 to 7 represent data recorded at increasing levels of microwave power. The ambient temperature was measured as indicated in *Materials and Methods*. The abrupt change in the spectral parameter $2T_{\parallel}'$ at low microwave power occurs at the phase transition temperature (23°) of dimyristoyl lecithin.

lipid bilayer. However, additional considerations must be made in cases where solid and fluid lipid phases separate (28) at a given temperature and composition, as demonstrated for the data taken at 36° in Table 1, part B. At 36°, pure dipalmitoyl lecithin exists as a solid phase and the label used shows a relatively high order parameter (0.455). At the same temperature, pure dioleoyl lecithin is in a fluid state with a lower order parameter (0.241). In mixtures of the two lipids at 36°, solid and fluid lipid phases coexist, and the measured order parameters for these mixtures show that the fatty acid spin labels partition preferentially into the fluid lipid phases when a phase separation occurs.

TABLE 2. *Comparison of order parameters* for different fatty acid spin labels in chick embryo fibroblasts (CEF), mouse fibroblasts (3T3), and human erythrocytes*

Cell type	Order parameters for labels I(m,n)			
	I(10,3)	I(7,6)	I(5,10)	I(1,14)
Erythrocytes	0.65	0.56	0.45	0.22
CEF (normal and transformed)	0.58	0.42	0.21	
3T3	0.59			
3T3-Py$_6$	0.59			

* Spectra were recorded at 37-38°. The calculation of order parameters is described in the legend to Table 1. Given the limits of error of order parameters (±0.01), normal and transformed chick embryo fibroblasts showed no differences in order parameters.

Measurements of order parameter for spin labels in phospholipid–cholesterol mixtures (5) indicate that a change of about 5% in the mole fraction of cholesterol in membrane lipids may be detected. The possible effects of the membrane proteins on the measured lipid fluidity is harder to express quantitatively. However, from a plot of mole fraction rhodopsin in rhodopsin–lipid recombinants against order parameter (7), it may be estimated that a change of 15–20% by weight in the fraction of protein in the membrane could be detected as a change in lipid fluidity.

Certain experimental variables are potential sources of artifacts in these measurements. These include scan time, modulation amplitude, temperature, and microwave power. Proper use of microwave power is particularly important. Although the signal strength may be increased markedly by operation at the higher power levels available on commercial electron paramagnetic resonance instruments, the useful level of microwave power is limited in aqueous samples by sample heating and signal saturation. Microwave heating results from the sample in regions of the sample cavity where the electric field is not zero and occurs readily with samples in media of high dielectric loss, notably, in water. Significant changes in spectra were produced in some samples by as low as 30 mW of microwave power in the samples of whole cells used here. The transition temperature of dimyristoyl lecithin (23°) provides a convenient internal standard for determining the tolerable level of microwave power in a particular sample configuration (Fig. 1). As shown in Fig. 1, at maximum microwave power settings the temperature inside the sample tube may be 9° above the ambient temperature of the temperature controller. The degree to which the sample is heated is also quite sensitive to small changes in the ratio of lipid to water in the sample. For instance, in an experiment similar to that described in Fig. 1, but differing in that the ratio of lipid to water in the sample was lower by a factor of two, the temperature displacement at maximum power was 13°.

Lipid Chain Flexibility in Membranes of Intact Cells. Table 2 shows that the spin-labeled fatty acids are sensitive to both the type of cell and the position of the label on the fatty acid chain. Notable is the fact that at a given temperature the flexibility of lipid chains in fibroblast membranes is considerably greater with several labels than in the membranes of erythrocytes. This is of interest because lectin-induced aggregation of membrane glycoproteins has been observed on both transformed fibroblasts and trypsin-treated erythrocytes (trypsin does not produce a significant change in the flexibility of lipids in erythrocyte membranes).

Normal 3T3 mouse fibroblasts and several varieties of transformed mouse fibroblast have been used most often in comparative studies of the distribution and mobility of lectin-receptor sites on cell surfaces (1–3). The results of spin label measurements in mouse fibroblasts are presented in Tables 3 and 4. In these experiments, the accuracy of the reported order parameter is about ±0.01. The repeated measurements shown in Tables 3 and 4 do not reveal consistent differences in fluidity between normal and transformed mouse fibroblasts.

Because a previous report of large differences in lipid fluidity in mouse fibroblasts has appeared (23), the data of Table 4 were collected using a spin label fatty acid of the

same structure as indicated in the other report and the same temperature of spectral measurement. When Fig. 2 is compared with the published spectra of similar experiments (23), one slight spectral difference is evident. The small signal (indicated by the arrow) arising from label dissolved in the aqueous medium in the spectra presented here is missing in the other reported spectra (23). If this spectral difference results simply from differences in the density of packing of cells in the samples, it should not be related to the observed order parameter differences that characterize the remaining, broad component of the spectrum. However, the lack of evident signal from label in water could also result if (*a*) the structure of the label used previously were incorrectly assigned or if (*b*) values of modulation amplitude or microwave power were considerably higher than those used in the present report. The most obvious way that the two differences between the present report and the previous one (23) (i.e., differences in order parameter and differences in amplitude of signal in aqueous medium) might be related is if very different microwave powers were used in the two studies. In one experiment with SVT_2 cells, the observed order parameter for label I(10,4) at 33° changed from 0.54 at 5 mW of power to 0.52 at 100 mW, indicating considerable elevation of internal sample temperature. Variability in the amount of microwave heating at 100 mW probably results from differences in water content or positioning of the sample in the resonance cavity. Also, the signal from label in water was nearly abolished (due to preferential saturation) at 100 mW.

DISCUSSION

The above data demonstrate that some of the inherent physical properties that describe the "fluidity" of lipids in normal and transformed cells may be quite similar even though differential mobility of cell surface lectin-binding proteins has been demonstrated in these same cells. Specifically, no significant differences were found in the order parameters of spin label fatty acids incorporated in the membranes of normal and transformed mouse fibroblasts. Differential agglutinability by lectins has also been demonstrated for chick embryo fibroblasts and RSV-transformed chick cells. Spin label measurements of lipid chain flexibility in normal chick fibroblasts, RSV-transformed chick fibroblasts, and chick fibroblasts infected with a temperature-sensitive RSV mutant (TS-68) have been reported (14, 15). Cells of each type were grown at 36° and 41°. Again, no differences in lipid flexibility were detected for normal and transformed cells. Also, although the cellular location of label is not defined in these experiments, measurements were usually made within several minutes of introduction of label. This, together with the rapid cellular destruction of label, indicates that a substantial portion of label is in plasma membrane.

It is not possible to make a detailed interpretation of the data presented here because little is known about how lateral mobility of membrane proteins is influenced by the variety of motions that contribute to what is termed "lipid fluidity." The fatty acid spin labels provide information specifically about the amplitude of motion and flexibility of lipid acyl chains. However, as used here, they do *not* measure directly other known characteristic features of fluid lipid bilayers, including lateral diffusion (30), phase separations (28), size and extent of fluid domains (31), and apparent microviscosity in the regions immediately surrounding membrane proteins. It is possible that other methods, which are sensitive to motions of different frequencies or which use other small

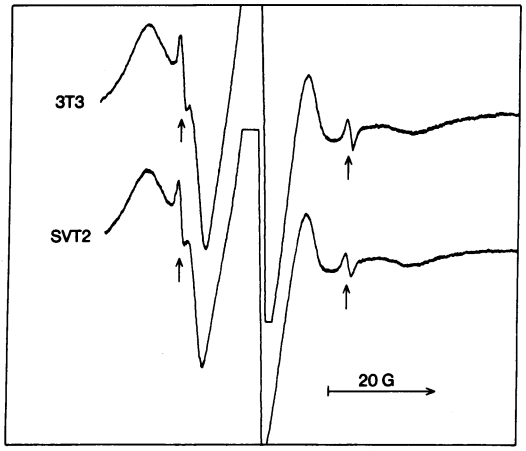


FIG. 2. Representative paramagnetic resonance spectra of label I(10,4) in the membranes of normal 3T3 and transformed SVT_2 mouse fibroblasts.

TABLE 3. *Order parameters* for label I(10,3) in whole mouse fibroblasts†*

Cell type	Order parameter	Observed‡ $T_{\parallel}'$	Observed‡ $T_{\perp}'$	Temperature of spectral measurement
3T3d	0.64	26.4	9.0	25°
	0.64	26.35	9.05	25°
	0.64	26.6	9.1	25°
Py_6	0.64	26.2	9.1	25°
	0.65	26.7	8.9	25°
	0.65	26.65	8.9	25°
3T3d	0.59	25.4	9.4	37°
	0.58	25.2	9.5	37°
	0.59	25.3	9.4	37°
Py_6	0.59	25.5	9.3	37°
	0.59	25.4	9.4	37°

* The calculation of order parameters is described in the legend to Table 1.

† Label was introduced by procedure A (*Materials and Methods*).

‡ Values are given in gauss.

TABLE 4. *Order parameters* for label I(10,4) in whole mouse fibroblasts*

Cell type	Order parameter	Observed† $T_{\parallel}'$	Observed† $T_{\perp}'$	No. of measurements averaged	Labeling procedure‡
3T3d	0.55	24.75	9.68	2	A
	0.55	24.9	9.80	1	B
SVT_2	0.55	24.7	9.60	2	A
	0.55	24.6	9.63	2	B
A_{31}	0.55	24.9	9.68	2	B
Py_6	0.54	24.4	9.70	2	B
Py_6R_1	0.54	24.5	9.75	2	B

* All spectral data were recorded at 33°. The calculation of order parameters is described in the legend to Table 1.

† Values are given in gauss.

‡ Procedures for introduction of the labels into cells are described in *Materials and Methods*.

molecular probes that take up entirely different orientations from those of fatty acids in bilayers, may reveal differences in some of the motions of lipids in the membranes of the normal and transformed cells studied here. It is also possible that very small and differential changes in order parameter might occur on interaction of these fibroblasts with certain drugs and effectors (10, 11).

There certainly is reason to believe that the mobility of membrane proteins *is* affected by lipid fluidity in some cases, although normal and transformed cells need not differ in this regard. The rates of both lateral and rotational diffusion of rhodopsin in rod disc membranes (32) are consistent with estimates of the lipid viscosity in other cell membranes (33, 34), the activities of membrane-bound enzymes are sensitive to lipid phase separations in bacterial membranes (35), and, of particular note to the results reported here, the ability of concanavalin A to agglutinate cells, which undoubtedly involves glycoprotein mobility, is dramatically affected by the lipid composition in mammalian cells provided with different fatty acid supplements (16). In the latter study, normal and transformed cells differed only slightly in the dependence of agglutination on lipid fluidity. Undoubtedly, a certain level of flexibility of lipid chains is required for lateral mobility of membrane glycoproteins. The factors resulting in *differential* mobility of lectin-binding glycoproteins in the normal and transformed cells discussed here remain to be determined but apparently need not include alterations in the physical properties of membrane lipids (36).

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