Alterations in Lipid Acyl Group Composition and Membrane Structure in Cells Transformed by Rous Sarcoma Virus†

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ABSTRACT: The acyl group composition of the phospholipids from normal chick embryo fibroblasts and from cells transformed by Rous sarcoma virus was determined by gas-liquid chromatography. Rous-transformed cells had less arachidonate (20:4) and more oleate (18:1) in membrane lipids than normal, growing cells. Normal density-inhibited cells had the lowest ratio of 18:1/20:4. Associated with the decreased content of 20:4 in the transformed cells was a decreased motional freedom of an incorporated spin-labeled fatty acid analogue. Arrhenius plots for uptake of 2-deoxyglucose revealed an increased apparent activation energy in the transformed cells, suggesting that the hexose transport carriers were sensitive to the changes in membrane composition and structure in fully transformed cells. However, the development of the changes in fatty acid composition occurred relatively slowly in the course of transformation, indicating that the observed compositional alterations are not likely to be a primary cause of the early changes in membrane function associated with malignant transformation.

It is widely suspected that alterations in cellular membranes play a critical role in the expression of the malignant state. But although numerous transformation-specific changes in the chemistry and function of membranes have been reported (see Robbins and Nicolson, 1975, for a recent review), it is by no means clear how or whether the chemical changes affect membrane structure and function. Among the alterations in membrane activity associated with viral transformation are an increased rate of hexose transport (Hatanaka and Hanafusa, 1970; Weber, 1973) and an increased lateral mobility of lectin receptor sites (Robbins and Nicolson, 1975). Since the state of the membrane lipids has been shown to affect several transport systems and membrane activities (Wilson et al., 1970; Overath and Trauble, 1973; Grisham and Barnett, 1973; Kimelberg and Papahadjopoulos, 1972; and Rottem et al., 1970) and could reasonably be expected to affect the lateral mobility of membrane components (Horwitz et al., 1974), it seemed possible that virus-induced alterations in membrane composition and structure could underlie some of these functional changes.

It had previously been demonstrated that cells transformed by Rous sarcoma virus show little or no change in cholesterol content (Perdue et al., 1971b) or phospholipid composition and turnover (Quigley et al., 1971, 1972). We therefore examined the acyl group composition of the phospholipids from these cells to determine whether they are altered during malignant transformation, whether these alterations are associated with changes in bilayer structure, and whether the changes in membrane structure and chemistry could account for some of the changes in membrane activity. The results indicate a somewhat decreased unsaturation of some of the fatty acids in the transformed cells[1] and a corresponding small decrease in the motional freedom of an incorporated spin-labeled probe. However, studies on the kinetics with which the acyl group changes occur suggest that it is unlikely that these alterations in membrane chemistry and structure can account for the changes in membrane activity seen in the transformed cells.

Experimental Procedure

Cell Culture. Cell culture procedures and transport measurements were essentially as described previously (Weber, 1973). All infections were performed on primary cultures from 10-day C/O COFAL or CHF negative chicken embryos (SPAFAS, Roanoke, Ill.) using the Schmidt-Ruppin strain of Rous sarcoma virus, subgroup A at a multiplicity of approximately one. Primary or secondary cultures of chick embryo fibroblasts 4–7 days old were removed from the culture dish with 0.05% trypsin and replated on 100-mm plastic tissue culture dishes at the following densities: $0.5–2 \times 10^6$ cells/dish for the normal, exponentially growing cultures, $1.5–3 \times 10^6$ cells/dish for the transformed cultures, and $6–8 \times 10^6$ cells/dish for the density-inhibited cultures. The transformed and the normal growing cultures were changed to fresh growth medium daily, to keep both sets growing at the same rate and to minimize the pH drop that occurs in the transformed cultures. Cells were collected for analysis on day 2 or 3 after plating. From 4 to 8 dishes were used for each determination. The growth medium used in all these experiments was Dulbecco's Modified Eagle Medium (Grand Island Biological, Grand Island, N.Y.), supplemented with 10% Tryptose Phosphate Broth (Difco), 4% calf serum, and 1% heat-inactivated chicken serum. In previous experiments, density-inhibited cells were kept in a growth medium containing only 2% serum (Weber, 1973), since high serum retards the onset of density-dependent inhibition of growth (Holley and Kiernan, 1968). However, since the acyl group composition of cells may be affected by the free fatty acids and triglycerides supplied in the growth medium (Spector, 1972), we decided to compare only cultures grown in identical media, containing 5% serum. We also have analyzed the acyl group composition of normal and transformed cells grown in the low serum medium (2% Tryptose phosphate broth, 1% calf, and 1% chick serum) and have obtained results very similar to those reported here.

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[1] A preliminary report of some of this work has appeared (Yau and Weber, 1972).

Lipid Analysis. Growth medium was removed from the cells by aspiration and the culture was rinsed twice with cold phosphate-buffered saline (Weber, 1973). Cold methanol (2.5 ml) was added to each plate, and the cells were scraped off with a polyethylene policeman and transferred to a glass vial. Two volumes of chloroform was then added. The mixture was homogenized with a Teflon pestle. Two-tenths volume of water was added to the lipid extract for the partitioning of aqueous and organic phases (Folch et al., 1957). The aqueous phase was discarded and the organic phase was evaporated to dryness and taken up in a minimum volume of chloroform. Major phosphoglycerides (CPG,[2] GPE, and IPG + SPG) were separated on 0.1 M Na_2CO_3-impregnated silica gel G TLC plates with a solvent system containing chloroform–methanol–15 N NH_4OH (65:25:4, v/v). For the separation of ethanolamine plasmalogen from the diacyl type ethanolamine phosphoglycerides, the silica gel G spot containing total GPE was eluted with chloroform–methanol (1:1, v/v) and applied to another TLC plate. The TLC plate was exposed for 5 min to concentrated HCl fumes to cleave alk-1-enyl groups from the alkenylacyl GPE. The resultant monoacyl GPE and the HCl-stable diacyl GPE were then separated by TLC. Lipid spots on silica gel G plates were visualized under ultraviolet light after spraying the TLC plates with 2′,7′-dichlorofluorescein reagent. The acyl groups from total lipid extracts or from the individual phosphoglycerides were transesterified to fatty methyl esters by alkaline methanolysis (Horrocks and Sun, 1972). Conditions for the gas-liquid chromatographic analysis of methyl esters have been described previously (Sun and Horrocks, 1969). In the study of acyl group composition of the total lipid extract, the majority of the acyl groups were probably derived from the major esterified glycerides, such as CPG, GPE, IPG, SPG, diglycerides, and triglycerides. Free fatty acids and sphingomyelin are not reactive under these conditions.

Spin-Labeling Procedure. Spin-labels were purchased from Syva, Palo Alto, Calif. Cells were labeled with the lipid analogues by dissolving the labels to 10^{-1} M in absolute ethanol and dispersing this solution to a concentration of 10^{-4} in the growth medium that the cells had grown in for 12–18 h. The label was incubated with the cells for 6–8 h before harvesting. This long exposure time was chosen to maximize the uniformity of probe distribution in cellular membranes. After incubation, the medium containing the label was removed by aspiration and the cells were washed on the dish two times with 12-ml portions of cold phosphate-buffered saline. Cells were then scraped from the dish with a rubber policeman into 2.5 ml more of cold saline and centrifuged for 15 min at 10 000*g*. The saline was removed by aspiration, the sides of the tube were cleared of residual saline, and the cell pellet was used directly for the ESR measurements.

The fatty acid analogues and solvent, when exposed to cells for the time period and at the concentrations used in these experiments, did not affect the rates of growth or hexose transport of these cells.

ESR Measurements. Labeled cells from the above procedures were pooled from 4 to 16 100-mm culture dishes to give a sufficient quantity to fill a flat aqueous ESR sample cell. Spectra were run on a Varian E9, ESR spectrometer at room temperature (approximately 23 °C) at a microwave power of 5–80 mW, a modulation amplitude of 0.2–1.0 G, a time constant of 1.0, and a scan time of 4 min. In all experiments, care was taken to avoid power saturation and over-modulation effects, and paired samples were run at identical settings. No sample heating effects could be detected under these conditions, as measured with a copper–constantan thermocouple placed into the top of the sample.

Membrane Isolation. The procedure of Perdue et al. (1971a,b) was used for all membrane isolations. Cells were homogenized using a Teflon/glass homogenizer with a clearance of 0.002 in. (Glenco), and breakage was monitored in the phase microscope. Only the light "A" band fraction, which was cleanly separated from the other membranous fractions, was used in this work. This band was free from succinic dehydrogenase activity and was enriched two- to tenfold in ouabain-sensitive ATPase activity.

Results

Fatty Acid Composition of Phospholipids of Normal and Transformed Cells. In Table I is shown the fatty acid composition of the various phospholipid fractions extracted from growing normal and transformed cells: choline phosphoglycerides (CPG), serine plus inositol phosphoglycerides (SPG + IPG), diacylglycerophosphorylethanolamines (dGPE), and also the acyl groups from the ethanolamine plasmalogens (aGPE). Except for the plasmalogen acyl groups, which were characteristically low in saturated fatty acids, the ratio of saturated to unsaturated fatty acids was near unity for all of the phospholipids, in both normal and transformed cells. However, the phospholipids did show some acyl group specificity. Thus, the predominant fatty acids in CPG were 16:0 (palmitate) and 18:1 (oleate), whereas the predominant fatty acid in dGPE and in SPG + IPG was 18:0 (stearate). Choline phosphoglycerides had the fewest and aGPE had the greatest number of double bonds per acyl group. "Double bonds per acyl group" is referred to in this report as the "Unsaturation Index".

Comparing the fatty acids of transformed cells and of normal, exponentially growing cells, we found a substantial drop in the percent 20:4 (arachidonate) in the transformed cells, and a roughly equivalent increase in the percent 18:1. This trend held true for all of the phospholipids analyzed. Thus, although the ratio of saturated to unsaturated fatty acids remained approximately constant in the normal and transformed cells, the *degree* of unsaturation decreased in the transformed cells, as shown by the trend toward a decreased unsaturation index.

There were also some reproducible changes in fatty acid composition that were restricted to specific phospholipids. For example, the content of 18:2 in SPG + IPG was lower in normal than in transformed cells, as was the 20:3 content of aGPE.

Similar changes in fatty acid composition were detected in the total phospholipids of isolated plasma membranes (Table II). Although the magnitude of the change in plasma membrane fatty acid composition in this experiment was not as pronounced as that obtained with phospholipids from whole cells, the same trend was evident: the transformed cell plasma membranes showed a decreased percentage of 20:4, an increased percentage of 18:1, and a drop in the unsaturation index, compared to the normal cell plasma membranes.

In Table III is shown the fatty acid composition of total lipid extracted from density-inhibited cells, as well as that of normal growing cells and transformed cells. It can be seen that the arachidonate content of the density-inhibited cells was even higher than that of the exponentially growing normal cells, and

[2] Abbreviations used are: CPG, choline phosphoglycerides; SPG, serine phosphoglycerides; IPG, inositol phosphoglycerides; GPE, *sn*-glycero-3-phosphorylethanolamine; aGPE, acyl groups from ethanolamine plasmalogen (alk-1-enyl acyl GPE); dGPE, diacyl GPE; TLC, thin-layer chromatography; ESR, electron spin resonance; RSV, Rous sarcoma virus.

TABLE I: Acyl Group Composition[a] of Phosphoglycerides from Normal and Rous Sarcoma Virus-Transformed Cells.

Acyl Groups	CPG		SPG + IPG		aGPE		dGPE	
	Normal Cells	Transformed Cells	Normal Cells	Transformed Cells	Normal Cells	Transformed Cells	Normal Cells	Transformed Cells
16:0	28.4 ± 1.1	31.0 ± 1.0	5.2 ± 0.7	5.8 ± 0.9	7.2 ± 0.5	7.7 ± 0.2	6.8 ± 0.7	7.5 ± 0.3
18:0	17.2 ± 0.6	16.0 ± 0.6	46.0 ± 0.2	44.2 ± 0.4	6.9 ± 1.5	5.1 ± 0.8	41.5 ± 1.6	36.9 ± 0.5
18:1	28.6 ± 1.2	35.3 ± 0.9	14.6 ± 2.1	17.0 ± 1.9	7.6 ± 0.4	13.3 ± 1.8	14.1 ± 1.1	23.1 ± 1.7
18:2	10.3 ± 0.4	8.0 ± 0.3	5.8 ± 0.3	8.0 ± 0.4	4.9 ± 0.4	8.4 ± 0.6	8.8 ± 0.5	12.0 ± 0.4
20:3	2.7 ± 0.1	1.9 ± 0.2	6.1 ± 0.6	6.1 ± 1.0	1.8 ± 0.1	5.8 ± 0.1	2.6 ± 0.3	2.7 ± 0.4
20:4	6.2 ± 0.7	2.6 ± 0.3	14.7 ± 1.1	10.9 ± 1.2	38.4 ± 0.6	32.5 ± 0.6	18.7 ± 1.0	11.7 ± 0.6
22:4	1.9 ± 0.3	0.9 ± 0.2	2.7 ± 0.9	3.0 ± 0.3	13.2 ± 2.0	11.3 ± 0.4	2.6 ± 0.5	3.4 ± 0.8
22:5	0.9 ± 0.1	0.5 ± 0.2	2.2 ± 0.5	2.2 ± 0.6	10.8 ± 0.6	8.3 ± 1.5	2.4 ± 0.4	1.5 ± 0.2
22:6	1.1 ± 0.2	0.5 ± 0.1	1.9 ± 0.2	2.7 ± 0.1	9.8 ± 0.6	7.6 ± 0.3	3.0 ± 0.4	1.9 ± 0.2
% saturated fatty acids	45.6	47.0	51.2	50.0	14.1	12.8	48.3	44.4
Ratio 18:1/20:4	4.6	13.6	0.99	1.56	0.20	0.41	0.75	1.97
Unsaturation index[b]	0.95	0.75	1.28	1.24	3.25	2.92	1.43	1.25

[a] Each value is the mean of six determinations from two separate experiments, expressed as a weight percentage of the total. [b] Unsaturation index = average double bonds per acyl group. ± = SEM.

TABLE II: Acyl Group Composition[a] of Phospholipids Extracted From Whole Cells and Isolated Plasma Membranes.

Acyl Groups	Whole Cells		Plasma Membranes	
	Normal Cells	Transformed Cells	Normal Cells	Transformed Cells
16:0	21.1	26.1	27.7	33.1
18:0	24.2	21.9	27.7	24.6
18:1	36.4	38.2	26.9	29.5
18:2	10.1	9.9	11.1	8.1
20:4	8.2	4.0	6.6	4.8
% saturated fatty acids	45.3	48.0	55.4	57.7
Ratio 18:1/20:4	4.4	9.6	4.1	6.2
Unsaturation index[b]	0.84	0.70	0.71	0.61

[a] Single determination, expressed as a weight percentage of the total. [b] Double bonds per acyl group.

TABLE III: Acyl Group Composition[a] of Total Lipids from Growing, Density-Inhibited and Transformed Cells.

Acyl Groups	Normal, Density-Inhibited Cells	Normal, Exponentially Growing Cells	Rous Sarcoma Virus-Transformed Cells
16:0	17.0	20.9	16.8
18:0	31.8	20.6	24.3
18:1	15.6	23.8	30.7
18:2	9.2	12.6	10.4
20:3	1.3	3.2	3.6
20:4	22.6	14.5	10.1
22:4	1.1	1.8	1.8
22:5	0.6	1.2	1.0
22:6	0.8	1.6	1.4
Total saturated fatty acids	48.8	41.5	41.1
Ratio 18:1/20:4	0.7	1.6	3.0
Unsaturation index[b]	1.30	1.28	1.14
Hexose transport activation energy	10.9 kcal	10.6 kcal	14.2 kcal

[a] Each value is the average of two determinations, expressed as a weight percentage of the total. Error was less than 5%. [b] Double-bonds per acyl group.

the oleate content was correspondingly lower. However, the unsaturation index of these density-inhibited cells was identical to that obtained with the normal, exponentially growing cultures, since the increased percentage of 20:4 was compensated for by an increased percentage of 18:0.

Structural Correlates of the Acyl Group Changes. To determine whether the changes in acyl group composition alter the structure of the lipid bilayer, we measured the paramagnetic resonance spectra of a series of nitroxide-labeled stearic acid analogues that were incorporated into the growing cells. These analogues are of the general formula $I_{m,n}$, where m and n are the number of carbons separating the nitroxide from the methyl terminal and carboxyl terminal ends, respectively, of the fatty acid chain (Libertini et al., 1969; Hubbell and McConnell., 1969). The stearic acid analogues used here have a paramagnetic nitroxide at either the 5 ($I_{12,3}$), 12 ($I_{5,10}$), or 16 ($I_{1,14}$) position of the polymethylene chain and have been shown to monitor the environment in different regions across the membrane in cases where the lipids are thought to be arranged in a bilayer (Keith and Melhorn, 1972; Hubbell and McConnell, 1971), the motional freedom of the $I_{1,14}$ label being most sensitive to the environment in the center of the bilayer.

In Figure 1 are shown the ESR spectra of the three stearate labels incorporated into normal exponentially growing cells (A) and Rous sarcoma virus transformed cells (B). As the radical moiety is moved closer to the carboxyl end of the fatty acid, these spectra reflect a decreased motional freedom of the hydrocarbon chain to which the radical is attached. This presumably is because the fatty acid analogue is incorporated into a membrane bilayer where the carboxyl end is immobilized and the hydrophobic end of the polymethylene chain is less restricted in its motion. To quantitate simple three line spectra,

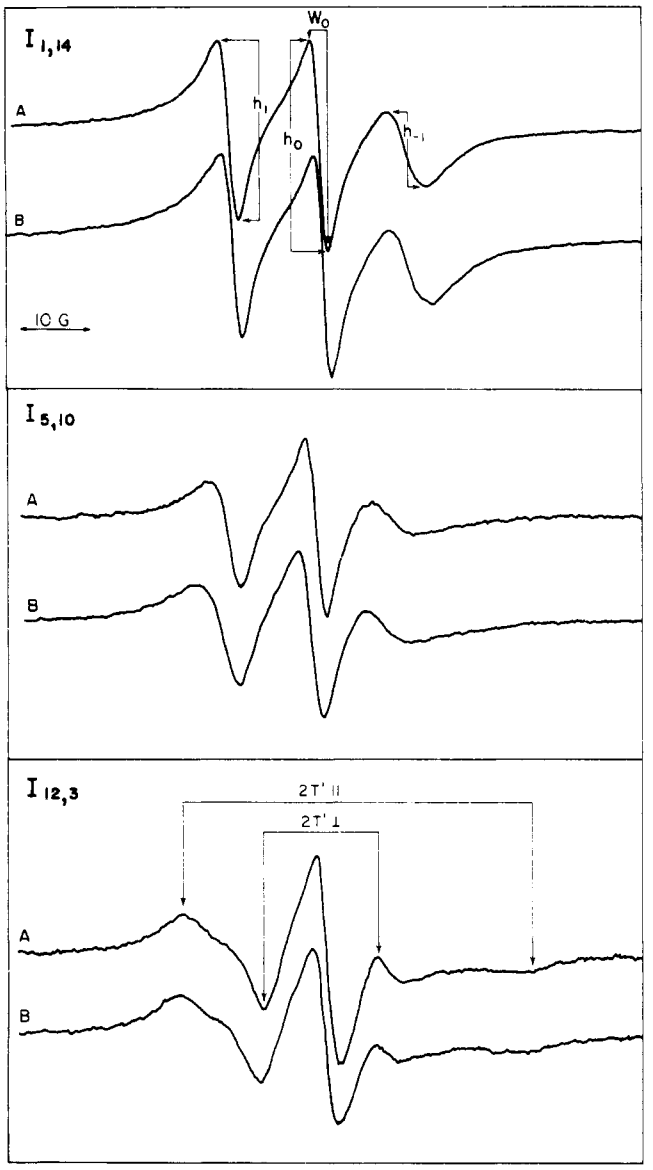

FIGURE 1: ESR spectra of spin-labeled stearic acid analogues incorporated into (A) normal growing cells and (B) RSV-transformed cells.

TABLE IV: Typical[a] Spectral Data and τ_c Values.[b]

Cell Type	W_0 (G)	h_0/h_1	h_0/h_{-1}	τ_c (s × 10^{-10})
		$I_{1,14}$		
Normal, growing	3.0	1.21	2.78	14.9
Transformed	3.0	1.23	2.99	16.2
		$I_{5,10}$		
Normal, growing	3.4	1.68	5.49	36.0
Transformed	3.9	1.66	5.25	39.7
	$T'_\perp$	$T'_\parallel$	a/a'	S
		$I_{12,3}$		
Normal, growing	9.9	27.3	0.90	0.63
Transformed	10.0	28.1	0.88	0.64

[a] Data taken from single experiments, in which cells in each of the physiological states were simultaneously labeled with the stearic acid analogue. [b] Rotational correlation times (τ_c) were calculated as described by Sinensky (1974), based on the formulation of Stone et al. (1965). $\tau_c = 6.45 \times 10^{-10} W_0 (h_0/h_{-1})^{1/2} + (h_0/h_1)^{1/2} - 2)$. [c] Order parameters ($S$) were calculated according to Hubbell and McConnell (1971): $S = ((T'_\parallel - T'_\perp)/(T_{zz} - T_{xx}))(a/a')$. T_{zz} and T_{xx} were taken as 30.8 and 5.8 G, respectively, $a' = 2T'_\perp + T'_\parallel$; $a = 2T_{xx} + T_{zz}$. As suggested by Hubbell and McConnell, 0.8 G was added to the measured values of $T'_\perp$.

TABLE V: Differences between Cell Types in τ_c and S Values for Incorporated Spin-Labels.[a]

Label	$\tau_c^T - \tau_c^N$
$I_{1,14}$	1.7 ± 0.5 (12)
$I_{5,10}$	2.9 ± 1.4 (4)
	$S^T - S^N$
$I_{12,3}$	0.02 ± 0.01 (3)

[a] τ_c^T, S^T, τ_c^N, S^N, = τ_c or S for transformed or normal growing cells, respectively; τ_c = s × 10^{-10}, ± = standard error. Values in parentheses are the numbers of independent experiments used in calculating the mean and the standard error. The data include the measurements made on cells infected with the temperature-conditional mutant, RSV-T5 (Martin, 1970) and on isolated plasma membranes (Perdue, 1971a,b).

such as obtained for $I_{1,14}$ and $I_{5,10}$, an approximate rotational correlation time (τ_c) assuming isotropic tumbling, can be calculated as described in the legend to Table IV. Since the expression used to calculate τ_c does not take into account anisotropy of the tumbling of the bound nitroxide (which will also affect relative widths and heights of the spectral lines) the absolute values of τ_c obtained are very approximate (especially in the case of $I_{5,10}$), but should nonetheless reflect *relative* mobilities of the labels in each case. An increased value of τ_c reflects a decrease in the motional freedom of the nitroxide.

The method used to calculate τ_c is not applicable for spectra from more immobilized labels, such as $I_{12,3}$. Therefore, to quantitate spectra from cells labeled with $I_{12,3}$ we calculated the "order parameter" (S) as described by Hubbell and McConnell (1971) (see legend to Table IV). High values of the "order parameter" are characteristic of relatively immobilized probes, whereas low values indicate that the probe is more mobile.

Effect of Cellular Changes on the ESR Spectra of Bound Labels. When ESR spectra of each of the stearic acid labels were recorded for normal growing and transformed cells, small changes in motional freedom of the labels were observed, which are summarized in Tables IV and V. Although there was substantial variability from experiment to experiment in the absolute values obtained (which seems not be uncommon in biological systems (Butterfield et al., 1974)), the *differences* between cell types were qualitatively reproducible in replicate experiments performed over a period of more than a year (Table V). Label mobilities are given as τ_c for $I_{1,14}$ and $I_{5,10}$ and are given as S for $I_{12,3}$. Only the normal and transformed cell pairs labeled with $I_{1,4}$ were examined often enough for a convincing statistical analysis. The difference in τ_c between these pairs was found to be reliable at $p < 0.01$ using a two-tailed Student's t test. These data clearly show a decreased mobility of the label in the transformed cells, compared to the normal, growing cells, consistent with the idea that a decreased unsaturation index would allow better packing of the lipid acyl groups and give rise to a decrease in their motional freedom. The decreased mobility of incorporated $I_{1,14}$ in transformed cells was also observed in isolated plasma membrane preparations (Perdue et al., 1971a,b) and in cells transformed by the temperature-conditional mutant RSV-T5 (Martin, 1970) (data not shown). The data obtained with the other spin labels

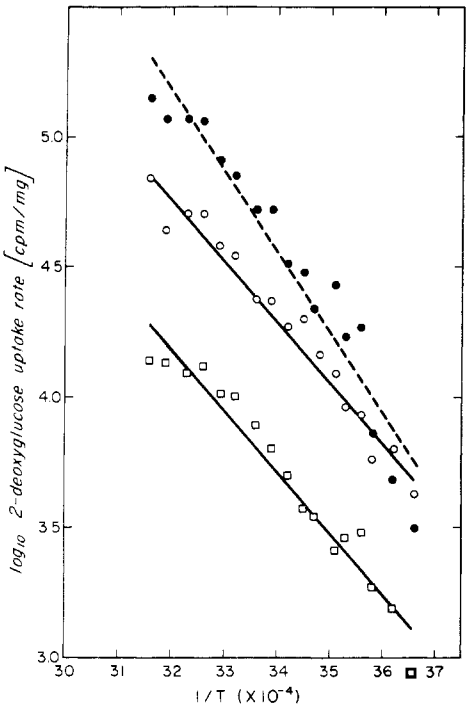

FIGURE 2: Arrhenius plots for 2-deoxyglucose uptake by density-inhibited (□—□), normal growing (○—○), and transformed cultures (●—●).

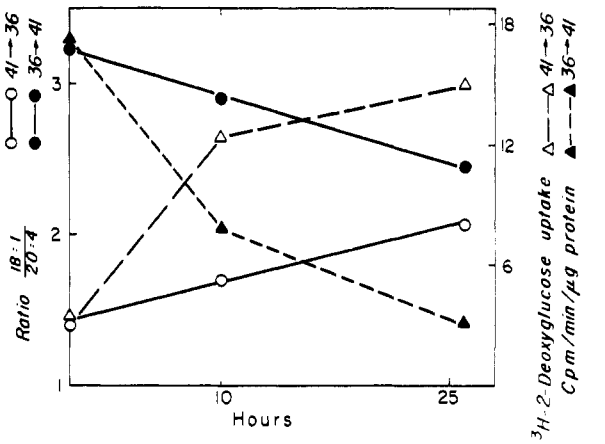

FIGURE 3: Kinetics of change in 2-deoxyglucose uptake (triangles) and ratio of 18:1/20:4 in whole cell total lipids (circles) in cells infected with RSV-T5 following a shift in temperature from 36 to 41 °C or from 41 to 36 °C.

can only be considered corroborative and suggestive and are not statistically significant.

Arrhenius Plots for Hexose Transport. Maintenance of a "fluid" lipid phase is important for a number of membrane-associated enzymatic activities, including sugar transport in bacteria (Wilson et al., 1970; Overath and Trauble, 1973), and Na/K dependent ATPase in animal cells (Grisham and Barnett, 1973; Kimelberg and Papahadjopoulos, 1972). At the temperature of transition from a more fluid to a more gel-like membrane state these reactions display a marked increase in the apparent Arrhenius activation energy. The temperature at which the phase transition occurs is dependent in part on the fatty acid composition of the membrane, occurring at a low temperature when the fatty acids are more unsaturated, and at a higher temperature when the fatty acids are saturated. We therefore expected that, if hexose transport carriers were sensitive to the alterations in fatty acid composition, the temperature-dependence of the transport reaction would be altered, the apparent Arrhenius activation energy being higher in the cells with the less "fluid" bilayer. We therefore measured the initial rate of uptake of 2-deoxy[^{3}H]glucose as previously described (Weber, 1973) at temperatures from 43 to 0 °C, and used the data to construct Arrhenius plots as shown in Figure 2. The lines were drawn by the least-squares procedure, using a Wang computer. One can see that the slope of the Arrhenius plot is steeper for the transformed cultures than for the normal cultures. There were no obvious or reproducible inflections in the temperature dependence, suggesting that a lipid phase transition did not occur sharply in the region of the hexose transport carriers. (The slight break in the transformed cell curve at 8 °C was not reproducible.) The absence of a sharp lipid phase transition is typical for complex, cholesterol containing membranes (Kimelberg and Papahadjopoulos, 1972; Marsh and Smith, 1973; Ladbrooke et al., 1968a,b). A similar linear change in the temperature dependence of enzyme activity with changed fatty acid composition has also been reported for the 1-acylglycerol-3-phosphate acyltransferase activity in *Escherichia coli* (Mavis and Vagelos, 1972). The apparent Arrhenius activation energy for hexose uptake, calculated from the slopes of the lines in Figure 2, is shown in Table III. (For technical reasons the uptake measurements could not be performed at saturating substrate concentrations, so these values cannot be considered true activation energies.) It can be seen that the apparent Arrhenius activation energy for the uptake reaction is inversely correlated with the unsaturation index of the membrane lipids, being higher in the transformed cells. Bose and Zlotnick (1973) have obtained qualitatively similar results for 3T3 cells transformed by Murine sarcoma virus: they obtained an apparent activation energy for 2-deoxyglucose uptake of 3.6 kcal/mol in the normal 3T3 cells and 7.4 kcal/mol in the MSV-transformed 3T3 cells. These findings are consistent with the notion that the hexose transport carriers are sensitive to the alterations in membrane lipid acyl groups.

Kinetics of Appearance of Fatty Acid Changes. To examine the time course of appearance of the tumor virus-induced fatty acid alterations, we used the temperature-conditional mutant of Rous sarcoma virus, RSV-T5 (Martin, 1970). Cells infected by this mutant are transformed at 36 °C and phenotypically normal at 41 °C, but produce infectious virus at both temperatures. In Table VI and Figure 3 the time courses of appearance of the fatty acid alterations are correlated with the changes in the rate of hexose transport which accompany transformation. Chick cells infected with RSV-T5 and held at 36 °C had a high rate of hexose transport and a fatty acid composition typical of transformed cells, whereas the cultures held at 41 °C displayed a normal, low hexose transport rate and a normal-type fatty acid composition. At time zero, cultures were shifted to the opposite temperature. At 10 and 26 h after the shift, cultures were taken for fatty acid analysis and for measurement of their capacity to take up 2-deoxyglucose. It can be seen (Figure 3) that, whereas the switch in the capacity to take up 2-deoxyglucose was complete within 26 h (as shown previously (Martin et al., 1971)), the changes in fatty acid composition were only half complete. The change in fatty acid composition occurred no faster in individual phospholipids (Table VI) or in the isolated plasma membranes (data not shown). Thus, the functional alteration in the cell membrane that is characteristic of cells transformed by RNA tumor viruses precedes the completion of the gross compositional alterations. Uninfected cells, cells infected with wild-type Rous sarcoma virus, or cells infected with a nontransforming mutant of Rous sarcoma virus showed no temperature-dependent

TABLE VI: Kinetics of Appearance of Acyl Group Alterations in Cells Infected with a Temperature-Conditional Mutant of Rous Sarcoma Virus.[a]

	41 → 36 °C			36 → 41 °C		
Acyl Groups	0 h	10 h	26 h	0 h	10 h	26 h
			Choline Phosphoglycerides			
16:0	31.0	27.8	28.9	29.3	30.6	29.9
18:0	16.3	17.7	16.5	16.6	15.4	17.0
18:1	27.0	28.1	31.3	35.8	34.7	35.0
18:2	10.4	12.6	11.9	11.0	11.9	10.8
20:3	3.9	3.5	3.5	2.8	2.4	2.3
20:4	7.6	7.4	4.4	2.4	2.6	3.9
22:4	0.4	0.4	0.8	0.1	0.2	0.4
22:5	1.3	0.9	1.1	0.8	0.9	1.2
22:6	2.3	1.6	1.6	1.2	1.3	1.5
Ratio 18:1/20:4	3.6	3.8	7.1	14.9	13.3	9.0
Unsaturation index	1.13	1.00	0.93	0.84	0.82	0.87
			Serine Plus Inositol Phosphoglycerides			
16:0	5.3	4.8	3.5	3.9	3.0	3.2
18:0	44.0	44.0	44.8	44.5	43.7	44.6
18:1	12.4	14.5	13.7	20.7	15.8	16.2
18:2	5.2	6.6	6.9	8.5	7.5	6.7
20:3	5.8	7.1	7.1	5.5	8.7	7.2
20:4	19.5	13.6	14.5	9.8	14.6	16.3
22:4	2.9	2.8	3.2	1.6	1.4	1.9
22:5	2.4	3.4	3.4	2.5	2.2	1.8
22:6	2.6	3.2	2.8	3.0	2.9	1.9
Ratio 18:1/20:4	0.6	1.1	0.9	2.1	1.1	1.0
Unsaturation index	1.46	1.40	1.43	1.21	1.39	1.36
			Ethanolamine Phosphoglycerides			
16:0	5.3	5.2	5.0	6.4	5.7	5.3
18:0	27.6	29.1	29.5	29.7	27.9	29.3
18:1	11.7	12.8	14.4	19.3	17.2	17.5
18:2	8.5	8.3	11.2	13.3	12.0	11.0
20:3	5.2	4.9	5.9	5.1	4.5	2.7
20:4	24.4	23.5	20.1	17.0	17.7	21.5
22:4	3.4	4.6	4.2	1.2	2.0	2.9
22:5	4.1	4.4	4.2	3.1	3.6	3.7
22:6	10.0	7.2	5.5	5.1	9.4	6.1
Ratio 18:1/20:4	0.5	0.5	0.7	1.1	1.0	0.8
Unsaturation index	2.17	2.06	1.88	1.67	1.93	1.86

[a] Each value is the average of two determinations, expressed as a weight percentage of the total. Error was less than 5%.

changes in fatty acid composition over this temperature range (data not shown).

Discussion

The results presented here demonstrate that chick cells transformed by Rous sarcoma virus have less arachidonic acid and more oleic acid in their cellular lipids than do their normal, untransformed counterparts. This is true even when comparison is made between transformed cells and normal cells that are multiplying at the same rate. Moreover, cells infected with the RSV-T5 temperature conditional mutant of Rous sarcoma virus and held at the permissive temperature have the high 18:1/20:4 ratio characteristic of transformed cells, but when held at the nonpermissive temperature, they display a "normal" fatty acid composition, even though they produce virus at both temperatures. Thus, the change in fatty acid composition seems to be an intrinsic property of the transformed state, not contingent on changes in growth rate or on viral infection. Since mouse 3T3 cells and human WI38 cells transformed by SV40 virus show similar changes in acyl group composition (Horwitz et al., 1974; Howard and Kritchevsky, 1969) this alteration in lipid composition may be a universal characteristic of virally transformed cells. Rat hepatoma cells have also been reported to display a higher 18:1/20:4 ratio than normal liver (Veerkamp et al., 1962; Van Hoeven et al., 1975). We also have shown that, when normal cells become density inhibited, they display an additional drop in the ratio of 18:1/20:4. However, since this change is also accompanied by an increase in the percentage of saturated fatty acids, the unsaturation index of the density-inhibited cells remains the same as that obtained with the normal, exponentially growing cells.

Similar changes in fatty acid composition were seen in lipids extracted from whole cells and in lipids from isolated plasma membranes, suggesting that all the cellular membranes were altered to some extent. The data for lipids extracted from whole cells represents a weighted average value for the total cellular membranes that will be affected by the degree of change of each membrane fraction, and by the percentage of total membranes that that fraction constitutes.

Associated with the increased ratio of 18:1/20:4 in the transformed cells was a decreased motional freedom of an incorporated nitroxide-labeled stearic acid analogue. For the following reasons, we feel that the spin-labeled probes we used were in fact incorporated into cellular membranes, and, thus, that the spectral changes we observed reflected changes in membrane structure:

(1) The isotropic hyperfine splitting, a quantity which increases with solvent polarity for the nitroxides, was 14.2 G for the $I_{1,14}$ and $I_{5,10}$ labels and 15.7 G for $I_{12,3}$, as compared to 16.0 G for each of the free labels dissolved in growth medium. This is consistent with the incorporation of the labels into a bilayer membrane.

(2) The spectra of the bound labels were similar to those observed by other workers for lipid spin-labels bound to whole eucaryotic and procaryotic cells, purified membrane preparations, and artificial lipid bilayers, and very different from those of the free label (Kaplan et al., 1973; Gaffney, 1975; Butterfield et al., 1974; Sinensky, 1974).

(3) When a large excess of label was added to the growth medium refractile globules (presumably lipid) were seen in the cytoplasm, coinciding with the appearance of a spectrum of free label superimposed on the spectrum of bound label, probably due to excess label in the cytoplasm.

(4) The observed spectral changes correlate well with the expected effects of changes in fatty acid composition for the various cell types and with the expected environments of the three labels in the different regions of the membrane.

(5) A plasma membrane fraction prepared from normal and transformed cultures labeled with the stearic acid analogues showed spectral changes qualitatively similar to those seen with labeled whole cells. This result indicates not only that the probes were monitoring membrane structure, but that the structural changes occurred in the plasma membrane.

We interpret the spectral differences observed between the different cell types as indicating that the motion of the incorporated $I_{1,14}$ label was more restricted in the transformed cells than in the normal cells, and thus that the lipid acyl groups in the interior of the transformed cell membrane were slightly *less* "fluid" than those of normal cell membranes. These findings can be readily understood in terms of the fatty acid composition of these cells, since the lipids of the transformed cells have a lower percentage of arachidonic acid (20:4) and an increased percentage of oleic acid (18:1) than the lipids of normal cells. Thus, the motional freedom of incorporated $I_{1,14}$ varied with the percentage of arachidonic acid in cellular lipids, presumably due to the "fluidizing" effect of the long-chain polyunsaturated fatty acid. The cholesterol content and phospholipid composition of membranes from these cells does not change upon transformation by Rous sarcoma virus (Quigley et al., 1971, 1972; Perdue et al., 1971a) and thus cannot be responsible for the observed changes in membrane lipid "fluidity".

It is important to point out that interpretation of the ESR data reported here must be hedged by our uncertainty concerning the precise location of the probe within the membrane. The fact that we labeled our cells for 8 h with the probe and that isolated plasma membranes and whole cells both showed similar changes argues that we are measuring some average membrane property. But our results are undoubtedly sensitive to some extent to the distribution of the probe in various membrane domains and the degree of structural alteration in these domains.

The functional significance of the small alterations in membrane chemistry and structure we have detected is by no means clear. Transformed cells are more readily agglutinated by plant lectins than are normal cells, a phenomenon that seems to be related at least in part to increased mobility of lectin binding proteins in the plane of the membrane bilayer (Robbins and Nicolson, 1975). It has been suggested that increased lateral mobility of lectin binding sites might be caused by increased average "fluidity" of the membrane bilayer but the results presented here demonstrate that changes in motion of incorporated spin-labeled fatty acids that occur upon transformation are very small and are in a direction opposite to that expected: tranformed cells, which presumably have the highest binding-site mobility, also seem to have the lowest fatty acid "fluidity". These results are inconsistent with simple notions that gross changes in the "fluidity" of the lipid bilayer are responsible for transformation-specific changes in the lateral mobility of lectin binding sites. However, if the changes in membrane "fluidity" occur nonuniformly, or if they are associated with phase separations (Shimshick and McConnell, 1973; Linden et al., 1973) then it is conceivable that they may be involved in alterations in the lateral mobility of membrane proteins. However, the fact that normal and transformed cells display a nearly identical temperature dependence of lectin agglutinability (Horwitz et al., 1974) argues against any involvement of membrane lipid acyl group composition in causing the transformation-specific differences in agglutinability.

Cultured fibroblasts also display changes in the rate of hexose transport that are associated with growth control and malignant transformation: density-inhibited cells display a very low rate of hexose transport, normal, exponentially growing cells display an intermediate rate, and transformed cells have a very high rate of hexose transport (Weber, 1973). Thus, the rate that these cells transport hexoses is directly proportional to the ratio of 18:1/20:4 and is inversely correlated with the measured "fluidity" of the lipid bilayer. Although transformation-specific changes in the temperature dependence of hexose uptake were detected, consistent with the notion that the hexose transport carriers in fully transformed cells were sensitive to the chemical and structural changes in the bilayer, we doubt whether the large changes in transport rate could be brought about by these small changes in membrane microviscosity and composition. Moreover, the fact that the changes in fatty acid composition occur more slowly than the change in hexose transport rate strongly suggests that the compositional changes are not causing the functional changes. Since all of the individual phospholipids changed their acyl group composition slowly, one cannot argue that one of the phospholipids, which might turn over more rapidly, is critical in controlling the activity of the hexose transport system. One might hypothesize that, although it takes longer for a fully "transformed" fatty acid composition to appear than it does for a fully "transformed" rate of hexose transport, a "threshold" exists for the activation of hexose transport by fatty acids: only a partial change in fatty acid composition might be necessary to cause a complete activation of hexose transport when RSV-T5-infected cells are shifted down from 41 to 36 °C. But this seems quite unlikely, since the restoration of a "normal" transport rate precedes the restoration of a "normal" fatty acid composition when RSV-T5 infected cells are shifted up from 36 to 41 °C. In fact, it seems possible to us that the increased transport of glucose could be responsible for the decreased unsaturation of the fatty acids, by causing an increased flux through the glycolytic pathway and, thus, perhaps an altered availability of substrates and cofactors for fatty acid biosynthesis.

A number of other groups have also investigated transformation-associated changes in membrane bilayer composition and structure, with varying results. For example, Fuchs et al. (1975) using a fluorescent probe found *decreased* lipid fluidity in transformed fibroblasts, in agreement with our results. Shinitzky and Inbar (1974), also using a fluorescent probe, found a dramatic increase in membrane "fluidity" in a malignant lymphoma cell line relative to normal lymphocytes, and

a corresponding drop in cholesterol content. Gaffney et al. (1974) and Gaffney (1975) found no difference in the motion-sensitive ESR spectra of spin-labeled fatty acids between normal and transformed fibroblasts, whereas Barnett et al. (1974, 1975) reported significant increased fluidity in the transformed fibroblasts. Whether differences in cell types, culturing technique, labeling procedure or motion-sensitive probe account for these varying results remains to be seen, but the fact that measured variations in the average "fluidity" of membrane lipids do not correlate with malignant transformation suggests that gross changes in the "fluidity" of membrane lipids cannot be a primary cause of the transformed phenotype. This is consistent with our finding that the changes in acyl group composition are relatively slow to develop.

The mechanism underlying the changes in membrane composition is unknown. A transformation-specific decrease in the desaturation of linoleic acid (18:2) as reported by Dunbar and Bailey (1975) or alterations in uptake of serum lipid could account for the results. These possibilities are currently under investigation.

In conclusion, we have found that fully transformed cultures of chicken embryo fibroblasts display a decreased unsaturation of membrane lipid acyl groups and a corresponding decrease in the flexibility of membrane lipid acyl groups measured using a spin-labeled fatty acid. Studies on the uptake and metabolism of fatty acids by normal and transformed cells and studies on the localization of these changes in different membrane regions and fractions are expected to shed light on their genesis and functional significance.

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