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Effect of Modification of Plasma Membrane Fatty Acid Composition on Fluidity and Methotrexate Transport in L1210 Murine Leukemia Cells[1]

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ABSTRACT

The plasma membrane lipid composition in L1210 murine leukemia cells was dependent upon the type of fat fed to the host animal. Plasma membranes prepared from cells grown in animals fed 16% sunflower oil contained 39% saturated, 14% monoenoic, and 41% polyenoic fatty acids. In contrast, those from cells grown in mice fed 16% coconut oil contained 39% saturated, 31% monoenoic, and 23% polyenoic fatty acids. Although there were 34% more unsaturated bonds in the membranes from the cells grown on polyunsaturated fat, there was no difference in the phospholipid or cholesterol content of the membranes. Studies with the spin label probes 5-nitroxide and 12-nitroxide stearic acid indicated that the order parameters at temperatures between 15 and 40° were higher in the membranes of cells from animals fed the coconut oil diet. The transition temperature of the approximate rotational correlation time in the region of 20° was 2.5-3.5° higher in the membranes isolated from the cells grown in the mice fed coconut oil. These findings indicate that the fluidity of the membranes was altered by the diet-induced modifications in fatty acid saturation. Appreciable differences were noted when transport of the antineoplastic drug methotrexate by intact L1210 leukemia cells from animals fed the sunflower or coconut oil diets was compared. Although the apparent V_{max} was not significantly different, the apparent K_m for transport by the cells from animals fed the sunflower oil diet was 2.90 ± 0.35 μM as compared to 4.10 ± 0.1 ($p < 0.02$) by cells from animals fed the coconut oil diet. Therefore, in the lower range of methotrexate concentrations, the cells having the more fluid membranes exhibited greater permeability to methotrexate. Taken together, these results indicate that the changes in membrane lipid structure and physical properties brought about by fatty acid modification are sufficient to affect the entry of an antineoplastic drug into the L1210 cell.

INTRODUCTION

The fatty acid composition of mammalian cell membranes can be modified experimentally. This can be accomplished in tissue culture by altering the lipid composition of the medium or in the intact animal by changing the dietary fat composition (2, 4, 11, 19, 20). These modifications are associated with changes in the physical and functional properties of the cell membrane. For example, Solomonson *et al.* (41) have shown that alteration of the membrane fatty acid composition in Ehrlich ascites tumor cells produces changes in the activation energy and transition temperatures for the membrane-bound Na^+-K^+-ATPase. Enrichment of LM cell membranes with certain fatty acids was associated with an increase in activity of membrane-bound adenylate cyclase (11). This difference disappeared when the enzyme was solubilized, suggesting that the alteration in activity was related to the lipid environment of the intact membrane. Furthermore, changes in fatty acid composition have a marked effect on the temperature dependence of agglutination by plant lectins in both transformed and nontransformed fibroblasts (21, 22), and they influence phagocytosis and pinocytosis in mouse macrophages (29). Transport also is influenced by changes in membrane fatty acid composition. In this regard, Wisnieski *et al.* (44) have shown that the transition temperature as measured with electron spin resonance probes corresponds to the transition temperatures of α-aminoisobutyric acid transport in cultured fibroblasts. A similar effect has been observed in Ehrlich ascites tumor cells, where changes in membrane fatty acid composition are associated with changes in membrane fluidity as detected with spin probes and in α-aminoisobutyrate transport (25).

Because of the potential importance to chemotherapy, it seemed worthwhile to investigate whether changes in membrane lipid composition have any influence on the transport of antineoplastic agents into a tumor cell. Our previous work with the L1210 murine leukemia cell suggested that it might be possible to modify the fatty acid composition of its plasma membrane by dietary modification (5). In the present study, we have demonstrated this experimentally and shown that these changes are sufficient to alter the fluidity of the membrane as detected with spin-labeled fatty acid probes. Furthermore, we have found that these membrane lipid modifications are associated with differences in the transport of an antineoplastic drug, methotrexate, into the intact cell.

MATERIALS AND METHODS

Male DBA/2 mice (14 to 16 g) (The Jackson Laboratory, Bar Harbor, Maine) were fed either a predominantly saturated fat diet (basal fat-deficient mixture supplemented with 16% coconut oil) or a diet rich in polyunsaturates (basal fat-deficient mixture plus 16% sunflower seed oil). The fat-deficient base (Teklad Test Diets, Madison, Wis.) is a semisynthetic mixture containing 59% food grade sucrose, 26% casein (extracted to remove fat), 10% cornstarch, 4% U.S.P. grade mineral mix,

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and 1% U.S.P. grade vitamin mix. The exact fatty acid composition of these diets after addition of the oils (Ruger Chemical Company, Inc., Hillside, N. J.) has been reported (2); briefly, the sunflower oil contains 70% linoleic acid, whereas the coconut oil diet contains 93% saturated fatty acids. After 4 weeks of feeding, 1×10^5 L1210 cells were injected i.p. into the mice, and the diets were continued during the 7-day tumor growth period. Cells were harvested, washed, and counted as described previously (6). Plasma membranes were isolated, using a modification of the method of Tsai *et al.* (43). Aliquots of 10^9 cells were disrupted in 10 ml of an isotonic homogenizing medium containing 0.5 M hexylene glycol, 1.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM Na_3PO_4 (43), by forcing 4-ml aliquots through a 25-gauge needle (1.6 cm long). The best results were obtained using 30 passages through the needle, at which point greater than 90% of the cells were disrupted and the breakdown of nuclei was minimal, as indicated by light microscopy. Following centrifugation at $1000 \times g$ for 1 min to remove nuclei and unbroken cells, the supernatant was placed on a discontinuous sucrose gradient (30 and 45%, v/v), and centrifuged at $23,000 \times g$ for 30 min to yield a crude membrane fraction. Purified plasma membranes were isolated from this fraction by centrifugation at $140,000 \times g$ for 18 hr on a 30 to 60% continuous sucrose gradient, using a Beckman L3-40 ultracentrifuge with a SW 27 swinging-bucket rotor. All membrane isolation procedures were carried out at 4°. ATPase activity was determined by the method of Solomonson *et al.* (41). The difference between the activities in the presence and absence of 2 mM ouabain was considered as the Na^+-K^+-ATPase (EC 3.6.1.3) activity and the remainder as the Mg^{2+}-ATPase activity. The P_i liberated in these assays was measured by the method of Fiske and Subbarow (12). 5'-Nucleotidase (EC 3.1.3.5) (43), succinic dehydrogenase (EC 1.3.99.1) (18), NADPH-cytochrome *c* reductase (EC 1.6.2.3) (42), phospholipids (33), and protein (28) content were also determined on some of the subcellular fractions. Electron microscopy of the isolated plasma membrane fraction was carried out as previously described (41). Statistical analyses were performed using the *t* test.

Lipids were extracted from the purified plasma membranes with $CHCl_3$:CH_3OH (2:1, v/v) (13). An aliquot of the lipid extract was subjected to alkaline hydrolysis with 1.2 N KOH in 80% ethanol for 60 min at 57° (1). Fatty acids in the saponifiable fraction were methylated (31), and the methyl esters were separated using a Hewlett-Packard 5710A gas chromatograph equipped with a 1.8-m 10% SP 2340 on 100 to 200 mesh Chromosorb column. Peaks were identified by comparison of retention times to those of standard fatty acids (Applied Science Laboratories, State College, Pa.; Supelco, Inc., Bellefonte, Pa.).

Additional aliquots of the membrane lipid extract were used for other determinations. Cholesterol was measured using a Hewlett-Packard 5700 gas chromatograph equipped with a 1.8-m 3% SP2250 on 100 to 200 mesh Supelcoport column (9). Cholestane was utilized as an internal standard. Phospholipid classes were separated by thin-layer chromatography (33), using plates prewashed in ethyl acetate and developed in $CHCl_3$:CH_3OH:CH_3COOH:H_2O (100:50:14:6). The segments of the plates corresponding to known standards were scraped, and the phospholipids were extracted using $CHCl_3$:CH_3OH (1:1, v/v).

For electron spin resonance studies, the fatty acid spin label was added to albumin by first dissolving 15 mg of 5- or 12-nitroxide stearic acid[4] (Syva Associates, Palo Alto, Calif.) in diethyl ether and evaporating under nitrogen to form a film on the bottom of the container (22). Five ml of 5% bovine serum albumin solution (Fraction V, fatty acid free; Miles-Pentex Laboratories, Elkhart, Ind.) in Krebs-Ringer bicarbonate buffer were added, and the mixture was stirred mechanically for 60 min at 20°. The insoluble albumin was sedimented by ultracentrifugation at $33,000 \times g$ for 30 min at 4°, and the supernatant solution was diluted with buffer before use. The spin label was incorporated into the freshly isolated plasma membranes by incubating the membranes with the albumin solution containing the spin label at 20°, and the labeled plasma membranes were washed 3 times with fresh buffer. This resulted in a molar ratio of spin label to phospholipid of 0.01 to 0.001, as determined by comparison of membrane spectra to a 0.1% pitch-in-KCl standard (24). Electron spin resonance spectra were determined immediately with a Varian-V 4502 x-band spectrometer equipped with a variable temperature accessory calibrated against a copper constantan thermocouple. Transition temperatures were obtained from Arrhenius plots of log ($\tau_0 \times 10^{10}$) *versus* temperature (where τ_0 is approximate rotational correlation time), fitted by the method of least squares. Two separate membrane preparations were studied with each spin label probe.

Drug transport experiments were performed by incubating 15 to 20×10^6 L1210 cells with [3',5',7-^{3}H]methotrexate (Amersham/Searle Corporation, Arlington Heights, Ill.) in 1 ml of Eagle's minimum essential medium (Grand Island Biological Company, Grand Island, N. Y.). Unlabeled methotrexate was kindly supplied by Lederle Laboratories, Pearl River, N. Y. All methotrexate was purified by column chromatography on DEAE-cellulose with a linear gradient of 0.1 to 0.4 M ammonium bicarbonate, pH 8.3 (32). The cell suspension and medium were equilibrated separately for 5 min at 37 or 0° before they were mixed together. Uptake was terminated by rapid addition of 8 ml ice-cold phosphate-buffered saline (6), followed immediately by centrifugation at $2000 \times g$ for 2 min at 0°. The resulting cell pellets were washed twice and dissolved in Soluene 350 (Packard Instrument Company, Downers Grove, Ill.). Dimilune 30 (Packard Instrument Company) scintillation fluid was added, and the samples were counted on a Beckman LS-3133T liquid scintillation spectrometer. In each experiment, the amount of drug taken up at 0° was subtracted from the uptake at 37° in order to correct for rapid nonspecific adsorption of the drug to the cell surface (17, 38, 39). All of the kinetic data were obtained using an incubation time of 5 min and substrate concentrations between 0.5 and 12 μM. The kinetic constants were calculated using a computer program (Los Alamos Publication LA-2367 and Addenda) in which the sum of the squares of the difference between observed and calculated velocities is minimized. Equal variance for the velocities was assumed. The present modification in Fortran IV was written by Dr. G. Gordon of Miami University (Miami, Ohio), and the kinetic subroutines were written by Dr. K. Sando of the University of Iowa. Since the exact contribution of diffusion is

[4] The complete chemical names of the spin label probes are: 5-nitroxide stearic acid, 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl; 12-nitroxide stearic acid, 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxyl.

unknown, the kinetic parameters that we have obtained are apparent constants for the carrier-mediated process.

RESULTS

Membrane Enzymatic and Ultrastructural Features. The enzyme specific activities of the purified plasma membranes and the crude homogenate are shown in Table 1. Na^+-K^+-ATPase, Mg^{2+}-ATPase, and 5'-nucleotidase were enriched 37-, 11-, and 26-fold, respectively, in the purified membrane fraction as compared to the crude homogenate. The specific activities and enrichment in the membrane fraction were similar for L1210 membranes isolated from both the coconut and sunflower oil diet groups. 5'-Nucleotidase activity is a useful marker for the plasma membranes of L1210 cells (43). Na^+-K^+-ATPase activity has been found in high specific activity in the membranes of many cells (8), and its enrichment in the L1210 plasma membrane fraction suggests that it is also a useful membrane marker for this leukemic cell. Succinic dehydrogenase activity, a mitochondrial marker, had a lower specific activity in the purified plasma membranes as compared to the crude homogenate. NADPH-cytochrome *c* reductase activity, a marker for the endoplasmic reticulum, had a 50% lower specific activity in the plasma membrane fraction as compared with the ribosome-rich band, which was present in the continuous gradient above the plasma membrane band. Electron microscopy of the plasma membrane fraction revealed a predominance of membrane vesicles of various sizes, and only an occasional ribosome was observed in these preparations.

Fatty Acid Composition and Lipid Content of Plasma Membranes. There was a considerable difference in the fatty acid composition of the L1210 plasma membranes when the cells were grown in animals fed the 2 different diets (Table 2). The major difference was in the proportion of polyenoic and monoenoic fatty acids. Membranes of the cells from animals fed the sunflower oil diet contained almost 2-fold greater proportions of polyenoic fatty acids than did those from animals fed the coconut oil diet. Conversely, the plasma membranes from cells grown in animals fed the coconut oil diet contained more than 2-fold greater proportions of monoenoic fatty acids. There was no appreciable difference in the content of saturated fatty acids in the 2 membrane preparations. Of the individual acids, the major differences were an enrichment of oleate in the coconut oil group and an enrichment of linoleate in the sunflower oil group. The average number of double bonds per fatty acid in the membranes from the cells grown in animals fed the sunflower oil diet was 1.57 ± <0.1 (S.E.) as compared to 1.17 ± 0.1 ($p < 0.05$) in those from the cells grown in animals fed the coconut oil diet. The average carbon atom chain length was only slightly greater (18.34 ± 0.1) in the membranes of the sunflower group compared to those of the coconut group (18.11 ± <0.1) ($0.1 < p < 0.2$).

The 2 membrane preparations did not contain appreciably different amounts of cholesterol or phospholipid relative to protein content, and the molar ratio of cholesterol to phospholipid was the same in both preparations (Table 3). In additional experiments, the phospholipids were separated by thin-layer chromatography (Table 3). Choline phosphoglycerides were the major species and comprised twice the percentage of any other phospholipid. Ethanolamine phosphoglycerides, sphin-

Table 1

Marker enzyme specific activities in homogenate and membrane fractions

L1210 cells which had been transplanted into mice fed a polyunsaturated fat-rich or a saturated fat-rich diet were harvested, washed, and disrupted. Nuclei and mitochondria were removed from this homogenate by low-speed differential centrifugation. Plasma membranes were isolated as a band by continuous sucrose gradient centrifugation as described in "Materials and Methods." A second particulate fraction, termed the upper gradient fraction, was isolated from the sucrose gradient as a band located above the plasma membrane band. Enzyme specific activity is shown as the mean ± S.E. of values from 4 separate preparations, except in the case of the upper gradient fraction, which is the mean of determinations on 2 separate preparations.

	Marker enzyme specific activities (nmol/min/mg protein)		
	Plasma membranes	Homogenate	Upper gradient fraction
Na^+-K^+-ATPase	218.0 ± 4.8	5.8 ± 0.8	79.9
Mg^{2+}-ATPase	532.7 ± 41.8	48.6 ± 4.2	291.1
5'-Nucleotidase	669.6 ± 89.9	25.0 ± 2.1	
Succinic dehydrogenase	2.7 ± 0.3	4.3 ± 1.0	
NADPH-cytochrome *c* reductase	10.4 ± 1.5	2.6 ± 0.2	21.5

Table 2

Fatty acid composition of plasma membranes of L1210 leukemia cells

Purified plasma membranes isolated from L1210 cells grown in mice fed either a polyunsaturated or saturated fat-rich diet were extracted with $CHCl_3$:CH_3OH (2:1, v/v), and the lipids were subjected to alkaline hydrolysis. The fatty acids contained in the saponifiable fraction were methylated, and the methyl esters were separated by gas:liquid chromatography with SP2340 on 100 to 200 mesh Chromosorb. The percentage composition is expressed as the mean ± S.E. of determinations on 3 separate membrane preparations.

	Sunflower seed oil diet (%)	Coconut oil diet (%)
Classes		
Saturated	38.8 ± 2.0	39.2 ± 0.7
Monoenoic	14.5 ± 1.5	30.7 ± 2.4
Polyenoic	40.8 ± 1.4	23.0 ± 3.2
Individual acids[a]		
<14:0	1.9 ± 0.3	1.5 ± 0.5
14:0	1.0 ± 0.7	0.9 ± 0.2
16:0	14.0 ± 1.6	14.5 ± 0.5
16:1	2.3 ± 0.5	3.4 ± 0.8
18:0	23.2 ± 1.0	23.3 ± 0.8
18:1	11.0 ± 1.7	24.3 ± 2.4
18:2	17.6 ± 1.6	8.1 ± 1.2
18:3	1.2 ± 0.5	0.9 ± 0.6
20:1	0.1 ± 0.1	1.4 ± 0.3
20:2	2.9 ± 0.4	0.5 ± 0.5
20:4	9.1 ± 2.2	8.7 ± 3.1
20:5	2.4 ± 1.7	0.6 ± <0.1
22:4	2.2 ± 0.2	0.6 ± 0.3
22:5	3.5 ± 0.2	3.6 ± 1.0
22:6	2.9 ± <0.1	1.1 ± 0.3
Others[b]	4.7 ± 2.2	6.6 ± 1.5

[a] Among the individual acids, the amounts of 18:1, 18:2, 20:1, 20:2, 22:4, and 22:6 are significantly different ($p < 0.02$) between the 2 membrane preparations. The fatty acids are abbreviated as number of carbon atoms:number of double bonds.

[b] Includes 20:3 and unidentified.

Table 3

Plasma membrane lipid content

Purified plasma membrane fractions were extracted with $CHCl_3$:CH_3OH (2:1). Cholesterol was measured by gas:liquid chromatography, using 3% SP2250 on 100 to 200 mesh Supelcoport column, and cholestane as the internal standard. Protein and phospholipids were measured as described in "Materials and Methods." These values are expressed as the mean ± S.E. of determinations on 4 separate membrane preparations. None of the values for the 2 membrane preparations are significantly different ($p > 0.05$). The major phospholipids were separated using thin-layer chromatrography. The segments of the silica gel corresponding to authentic standards were scraped and extracted with $CHCl_3$:CH_3OH (1:1), and the lipid phosphorus content was determined. Each determination required a pool of purified plasma membranes from 3 different preparations.

Lipid	Sunflower seed oil diet	Coconut oil diet
Lipid composition		
Cholesterol:protein (µg/mg)	128.0 ± 12.8	131.1 ± 14.1
Cholesterol:phospholipid (mol/mol)	0.40 ± 0.03	0.39 ± 0.01
Phospholipid:protein (µg/mg)	625.8 ± 23.1	661.3 ± 82.2
Major phospholipids (%)		
Choline phosphoglycerides	42.2	43.3
Ethanolamine phosphoglycerides	19.6	18.8
Sphingomyelin	16.6	15.3
Serine and inositol phosphoglycerides	16.6	15.3
Choline lysophosphoglycerides	5.0	7.3

gomyelin, and serine and inositol phosphoglycerides each represented about 15 to 20% of the total. There was no difference in the percentage composition of individual phospholipids in the membranes from each of the cell types.

Electron Spin Resonance Data. First-derivative electron spin resonance spectra from the spin-labeled plasma membranes were anisotropic over the temperature range studied, 15–40° (Chart 1). This was true for both 5- and 12-nitroxide stearic acid-labeled membranes from both types of cells. The order parameter, *S*, was calculated from these spectra as a quantitative indicator of membrane fluidity. It is a measure of the average angular deviation of the fatty acyl chain of the spin label probe at the nitroxide group from the average orientation of the fatty acids in the membrane (22, 37). An *S* value of 1.0 is characteristic of relatively rigid lipid environments, and lower values indicate more fluid lipid phases. An estimate of *S* was obtained using the relationship (3):

$$S = \frac{43.7\ \mathrm{G} - T_{\perp}'}{46.1\ \mathrm{G}} \times 1.723$$

For nitroxide spin labels in biological membranes, Gaffney (15) has shown that $2\ T\perp'$, the separation between inner hyperfine extrema (Chart 1), is inversely proportional to *S* over the entire range of order ($0 < S < 1$).

Order parameters calculated at 6 representative temperatures between 15 and 40° are shown in Table 4. The membranes from the cells grown in the animals fed sunflower oil had lower *S* values than those grown in the mice fed the coconut oil diet. This was true with both the 5- and 12-nitroxide stearic acid probes, indicating that the highly unsaturated sunflower seed oil diet produces cells with more fluid membranes than does the saturated coconut oil diet. In addition, different values for the order parameter were obtained with the 2 spin label probes. At each temperature, the order parameter obtained with the 5-nitroxide stearic acid probe was higher than the value obtained with the 12-nitroxide probe. This reflects the greater restriction of motion of the fatty acyl chains closer to the membrane surface (14, 19).

To monitor the temperature dependence of the freedom of motion of the spin probe, the approximate rotational correlation time, τ_0, was determined using the relationship developed by Keith *et al.* (26), $\tau_0 = 6.5 \times 10^{-10}\ W_0\ [(h_0/h_{-1})^{1/2} - 1]$. W_0 is the width of the midfield line, and h_0 and h_{-1} are the heights in G of the mid- and high-field lines, respectively. Increased label mobility occurs as the temperature is raised, and this produces a lower value of τ_0. Arrhenius plots of log τ_0 over the temperature range that was tested are shown for 5-nitroxide stearic acid-labeled membranes prepared from L1210 cells grown in

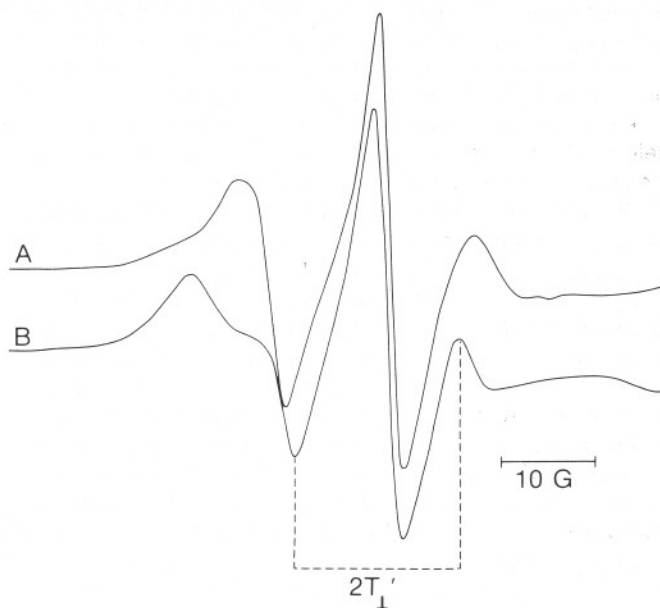

Chart 1. Representative electron spin resonance spectra of L1210 cell membranes spin labeled with 12-nitroxide (*A*) or 5-nitroxide stearic acid (*B*). These spectra are from the membranes of cells grown in animals fed the coconut oil diet and were recorded at 37°.

Table 4

Order parameters of electron spin resonance spectra

L1210 cells grown in animals fed the sunflower seed oil- or coconut oil-rich diets were harvested, and plasma membrane fractions were isolated following homogenization. The membranes were spin labeled with 5- or 12-nitroxide stearic acid. The electron spin resonance spectra over the temperature range 15–40° were recorded, and the order parameters, *S*, were calculated. The *S* values are the mean of determinations on 2 separate membrane preparations for each spin label probe.

	Order parameters (*S*)			
	12-Nitroxide stearic acid		5-Nitroxide stearic acid	
Temperature	Sunflower[a]	Coconut	Sunflower	Coconut
15°	0.578	0.620	0.671	0.682
20°	0.558	0.606	0.658	0.672
25°	0.541	0.562	0.638	0.650
30°	0.510	0.522	0.608	0.634
37°	0.460	0.465	0.572	0.603
40°	0.436	0.452	0.554	0.592

[a] Diet fed to the mice in which the cells were grown.

mice fed the 2 diets (Chart 2). Two transition temperatures were identified by discontinuities in these plots (34, 35). With the 12-nitroxide stearic acid probe, the lower transition temperature for the membranes of cells from animals fed the sunflower oil diet was 3.5° below that for the membranes of the cells from animals fed the coconut oil diet (18.5 *versus* 22.0°). Similarly, with the 5-nitroxide stearic acid probe, the lower transition temperature was 2.5° less for the membranes from the cells grown in animals on the sunflower oil diet (19.5 *versus* 22.0°). Both of these results are consistent with the order parameter calculations and indicate that the L1210 membranes from cells grown in animals fed the more unsaturated sunflower oil have a greater fluidity. The higher transition temperature was similar for membranes prepared from both types of cells, a result obtained with both probes (31.5 *versus* 31.5° with the 12-nitroxide stearic acid and 31.0 *versus* 31.5° with the 5-nitroxide stearic acid). The transition temperature at about 31° also failed to change with fatty acid modification of the Ehrlich ascites tumor plasma membrane, as determined by electron spin resonance (27). In summary, these results indicate that the fatty acid modifications produced in the L1210 cell membrane are sufficient to alter the fluidity of the plasma membrane, as measured with spin label probes.

Methotrexate Transport. The effect of these lipid modifications on membrane transport was assessed using methotrexate, a chemotherapeutic drug. Methotrexate was transported into the cells at an appreciable rate. Kinetic studies of initial uptake at 37° by cells from both diet groups remained linear for at least 10 min. As shown in Chart 3, the dependence of transport on methotrexate concentration was different in the cells derived from animals fed diets containing the 2 different types of lipid. The K_m' (apparent K_m) for the transport process was about 30% lower for the cells grown in animals on the sunflower seed oil diet, and this difference was statistically significant ($p < 0.02$) (Table 5). By contrast, the V_{max}' (apparent V_{max}) for the transport process was not significantly different in the cells from animals fed the 2 diets ($0.05 < p < 0.1$).

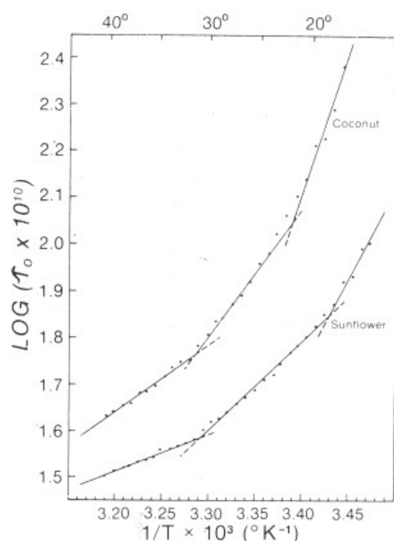

Chart 2. Arrhenius plots of the motion parameter τ_0 (in sec) of the 5-nitroxide stearic acid spin label probe in membranes from L1210 cells grown in animals fed the coconut or sunflower oil diets. The *lines* were fitted mathematically using the method of least squares. The correlation coefficient of the *points* forming each linear part of the plot was at least 0.98.

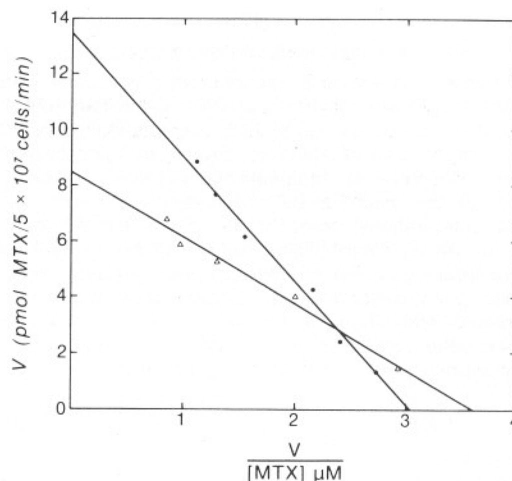

Chart 3. Eadie-Hofstee plots of the concentration dependence of methotrexate uptake. L1210 cells from mice fed the sunflower or coconut oil diets were incubated with [³H]methotrexate for 5 min. The *data points* were obtained by measuring initial velocity of cellular uptake at various substrate concentrations. The *lines* were fitted by the method of least squares and the correlation coefficients were 0.98 or greater. The abbreviations used are: *V*, initial velocity of cellular uptake; *MTX*, methotrexate. ●, coconut oil diet; △, sunflower oil diet.

Table 5

Apparent kinetic constants for methotrexate transport

L1210 cells were grown in animals fed either the 16% sunflower or coconut oil diets. The kinetic parameters were calculated, using a computer program which fitted the data to the Michaelis-Menten equation, and are expressed as mean ± S.E. of at least 4 separate experiments.

Diet	K_m' (μM)	V_{max}' (pmol/5 × 10^7 cells/min)
Sunflower	2.90 ± 0.35	9.31 ± 0.70
Coconut	4.10 ± 0.1	11.75 ± 1.08
p	<0.02	>0.05

DISCUSSION

These studies demonstrate that the lipid modifications in L1210 cells produced by diet are associated with changes in the plasma membrane fatty acid composition. The plasma membrane fraction prepared from the cells grown in mice fed the polyunsaturated sunflower oil diet contained almost 2-fold more polyenoic fatty acids and 34% more unsaturated bonds than those prepared from cells grown in mice fed the saturated fat-rich diet. The fatty acid modifications produced physical changes in the membrane as detected by electron spin resonance probes. Since there was no appreciable difference in the ratio of cholesterol to phospholipids, phospholipid head group composition, or fatty acid chain length, the alteration in fluidity in the L1210 membranes must have resulted primarily from the differences in fatty acyl saturation in the 2 membrane preparations. In addition, we have demonstrated differences in the transport of methotrexate, a chemotherapeutic drug, into these leukemic cells. The cells from the animals fed the polyunsaturated fat-rich diet had a lower K_m' for transport of methotrexate. Taken together, these data suggest that the differences in methotrexate transport are due to changes in the chemical and physical properties of the cell membrane lipids.

The transport of methotrexate by L1210 cells has been shown to be an energy-requiring, carrier-mediated process (7,

17, 38, 40). Therefore, the differences in transport that we have observed suggest that the properties and function of this carrier can be influenced by the fluidity of the membrane lipids in which it is embedded. Those cells that were enriched with polyunsaturated fatty acids and had more fluid membranes exhibited a lower K_m' for methotrexate transport. One explanation is that the structure of the carrier or of its binding site is affected by changes in the composition of the surrounding fatty acyl tails. Alternatively, the accessibility of methotrexate to the binding site of the carrier may be influenced by the surrounding lipid structure. The absence of any difference in V_{max}' suggests that the lipid alterations had no effect on the number of carrier molecules contained in the membranes of the 2 cell types. Results similar to these have been obtained for α-aminoisobutyric acid transport in lipid-modified Ehrlich ascites tumor cells (25). Increased membrane fluidity also was associated with a lower K_m' for α-aminoisobutyric acid transport but no change in V_{max}' for this process. α-Aminoisobutyrate transport is also energy dependent and carrier mediated. It would be of interest to determine whether other membrane carriers, like those for methotrexate and α-aminoisobutyric acid, are sensitive to changes in the fatty acyl composition of their environment.

Electron spin resonance has been validated as a technique to measure the fluidity of biological membranes (10, 14, 23, 34, 37). The 2 spin label probes that we used contained paramagnetic centers either 5 or 12 carbon atoms removed from the carboxyl terminus. These probes orient themselves in the membrane parallel to the acyl chains of the phospholipids. The spin labels then report the flexibility and degree of motion of the surrounding fatty acyl chains within the membrane. Sackmann *et al.* (36) have shown that the results obtained with a spin label probe in membranes may depend upon the position of the paramagnetic center within the membrane. The 5-nitroxide stearic acid probe provides information on order and motion at a relatively superficial level in the bilayer close to the phospholipid head groups. On the other hand, the 12-nitroxide probe senses conditions deeper within the lipid bilayer. This depth is below that to which the steroid nucleus of cholesterol molecules in the membrane bilayer extends. Our studies indicate that the 12-nitroxide stearic acid probe, which was located deeper within the lipid bilayer, reported a smaller order parameter. A similar finding has been observed in bilayer membranes and has been interpreted to indicate that the acyl chains have a greater degree of motion as the distance from the phospholipid head group increases (3, 14, 19, 22, 36). By contrast, we observed no major differences in the transition temperatures of the motion parameters, τ_0, obtained with the 2 spin label probes. This is in agreement with the findings of Morrisett *et al.* (30), who found similar membrane transition temperatures using 3 different physical probes. In the latter work, certain probes detected more transitions than others. In the present study, however, both probes detected only 2 transitions.

The L1210 leukemia cell is a model tumor system that is used extensively for preclinical screening of antineoplastic agents. Most drugs which are clinically useful have activity against the L1210 tumor; conversely, many agents with little activity against human neoplasms are inactive against this murine leukemia (16). The finding that the membrane lipid modifications that can be produced by diet during growth of this tumor are sufficient to influence methotrexate transport is of considerable potential significance. It suggests the possibility that the effectiveness of certain chemotherapeutic drugs may be modulated by changes in dietary fat composition. Since this could have therapeutic implications, it seems worthwhile to extend these observations to other important antineoplastic drugs.

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