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Acyl chain interactions and the modulation of phase changes in glycerolipids

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Fluorescence polarization measurements with 1,6-diphenyl-1,3,5-hexatriene and differential scanning calorimetry (DSC) were used to monitor phase transitions and order in the liquid state in sonicated dispersions of mono-, di- and triacylglycerols. Residual order in melted glycerolipids was indicated when the structural order parameter, S, assumed non-zero values at temperatures, $t \geq t_f$, the DSC-determined fusion temperature. Residual order was observed with *cis* unsaturated di- and triacylglycerols but not with corresponding *trans* unsaturated or with saturated compounds. The reduced fluidity was attributed to adjacent binding of fatty acids to the glycerol molecule and the resulting interactions between fatty acyl moieties and packing effects. Lipids were considered as in an isotropic liquid or highly fluid state when diphenylhexatriene fluorescence anisotropy, r_s, was equal to or less than 0.08, corresponding to $S = 0$. Temperatures, $t_{0.08}$, for transition from the fluid state upon cooling were noted when $r_s = 0.08$, and $\Delta t = t_{0.08} - t_f$ was then taken as a measure of residual order. Tri-, 1,2-di and 1,3-dioleoylglycerol Δt values were 75, 60.9 and 13.6°C, respectively. Tri-, 1,3-di- and monolinoleoylglycerol Δt values were 86, 30 and 41°C, respectively. Restrictions in mobility when observed are attributable to interactions between adjacent acyl chains. Double bond location in the hydrocarbon chain affected ordering in the liquid state as simple triacylglycerol esters of *cis* 18:1Δ6, *trans* 18:1Δ6 and *cis* 24:1Δ15 exhibited $t = 37$, 14 and 18°C, respectively.

Introduction

Thermal motion and dynamics of organization of lipids have been investigated intensively in the past several years with various physical techniques which occasionally yield disparate results. Attention has indeed been devoted to understanding why differences are noted when the thermotropic properties of some lipids and lipoproteins [1,2] determined by diphenylhexatriene fluorescence depolarization are compared with those determined by differential scanning calorimetry (DSC). The absence of diphenylhexatriene-evidenced phase transitions in human and animal lipoproteins has been attributed to probe locations [1,3] or to the heterogeneous nature of native lipoprotein fatty acids [2,4]. Berlin and Young [5] applied the theoretical interpretation of Van Blitterswijk et al. [6] to steady-state diphenylhexatriene anisotropy data for rabbit plasma lipoproteins, and they found evidence that lipoprotein core lipids undergo phase transitions to a highly fluid state, thus reconciling some differences between results of DSC and diphenylhexatriene fluorescence studies. In testing the methodology further with pure lipids we observed [7] by combined use of these techniques that *cis* fatty acyl unsaturation reduces probe mobility in melted triacylglycerols. The molecular

packing order parameter, S_{DPH}, determined from diphenylhexatriene (DPH) fluorescence anisotropy measurement was not zero when sonicated aqueous dispersions of trioleoyl- and trilinoleoylglycerol were held at temperatures equal to or higher than t_f their respective DSC-determined fusion temperatures. A zero value order parameter, typical of either an isotropic liquid or a highly fluid crystalline system, was expected for the melted triacylglycerols. The limitations on mobility in these triacylglycerols were attributed to acyl chain interactions.

The interpretation by Van Blitterswijk et al. [6] of diphenylhexatriene anisotropy was used to determine when $S_{DPH} = 0$. They developed a theoretical equation relating this structural order parameter to r_s, the steady-state anisotropy as:

$$r_s = \frac{2(1+2S)(1-S)(1-S^2)}{20(1+S)+5(1+2S)(1-S)} + r_0 S^2$$

where r_0 is the limiting anisotropy of the probe. According to this equation, $S_{DPH} = 0$ for $r_s \leq 0.08$, hence allowing definition [5] of a phase-transition temperature, $t_{0.08}$, as the temperature at which $r_s = 0.08$.

Though $t_{0.08} \gg t_f$ for triacylglycerols with *cis* unsaturated fatty acid moieties, $t_{0.08}$ was approximately equal to t_f for triacylglycerols with saturated or *trans* monounsaturated fatty acyl chains. This difference was associated with the molecular packing of the fatty acyl chains. The conformation of the saturated fatty acyl chains, usually represented by zigzagging polymethylene segments, is altered by twist angles introduced by *cis* double bonds in unsaturated fatty acids. It was proposed [7] that the restrictions introduced by the adjacent bonding of *cis* unsaturated fatty acyl chains to the same glycerol molecule results in limited fluidity even after melting, hence $r_s > 0.08$ at $t \geq t_f$. The concept that $t_{0.08} \gg t_f$ results from interactions between adjacent acyl chains and their molecular packing is now tested further with various isomeric glycerolipids and phospholipids. Several triacylglycerols were also examined to determine if double-bond position in the hydrocarbon chain affects these phase transitions.

Materials and Methods *

Lipids. Pure isomeric glycerolipids consisting of 1,2- and 1,3-dioleoyl (*cis* 18 : 1Δ9); 1,3-dielaidoyl (*trans* 18 : 1Δ9); 1,3-dilinoleoyl (*cis* 18 : 2Δ9,Δ12); monoelaidoyl; monolinoleoyl; tripetroselinoyl (*cis* 18 : 1Δ6); tripetroselaidoyl (*trans* 18 : 1Δ6); and trinervonoyl (*cis* 24 : 1Δ15) glycerols were purchased from Nu-Chek Prep, and used without further purification. 1,2- and 1,3-Dipalmitoyl (16 : 0) glycerol and the phospholipid isomers 1,3- and L-2,3-dipalmitoylphosphatidylcholine (DPPC) were purchased from Fluka and used without further purification.

Differential scanning calorimetry. Thermal analysis of lipid samples encapsulated in hermetically sealed aluminum capsules was conducted with the Perkin Elmer Model DSC-1B differential scanning calorimeter. Samples (0.5–3.0 mg) were transferred to the aluminum capsules in $CHCl_3/CH_3OH$ (2 : 1, v/v) and weighed with a microbalance following solvent removal in vacuo. Many of these lipids are subject to polymorphic transitions, therefore, all samples were cooled to at least 20°C below their lowest transition temperature using liquid N_2 as coolant and heated to at least 10 deg. C higher than the highest transition temperature. Calorimetry was performed on both anhydrous and hydrated samples and in both heating and cooling modes at scan-rates of 10 deg. C/min.

Fluorescence polarization. Fluorescence measurements were made with 1,6-diphenyl-1,3,5-hexatriene dissolved in lipids dispersed in aqueous media. Lipids, as thin films, were ultrasonically dispersed in 50 mM KCl with a Heat Systems Model W-225R Sonicator using a cup horn to avoid metal contamination. Diphenylhexatriene was incorporated by diluting 2 mM diphenylhexatriene in tetrahydrofuran 500–1000-fold into the lipid-containing dispersion before ultrasonic oscillation. The cup horn was maintained at a temperature higher than the appropriate melting temperature for each lipid sample as determined by DSC. Ultrasonic oscillation was continued for

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15 min at the maximum power output setting for the sonicator when used with a microtip. After sonication, the warm samples were transferred directly to cuvettes and fluorescence measurements were taken as a function of temperature beginning with a temperature at least several degrees above the highest DSC-determined melting temperature. Fluorescence measurements were taken as the samples were cooled in 4 C deg. increments. Each cooling step required 10–12 min for thermal equilibrium following manual adjustment of the temperature of the cuvette holder thermostat. Temperatures were monitored with a thermocouple immersed in the sample.

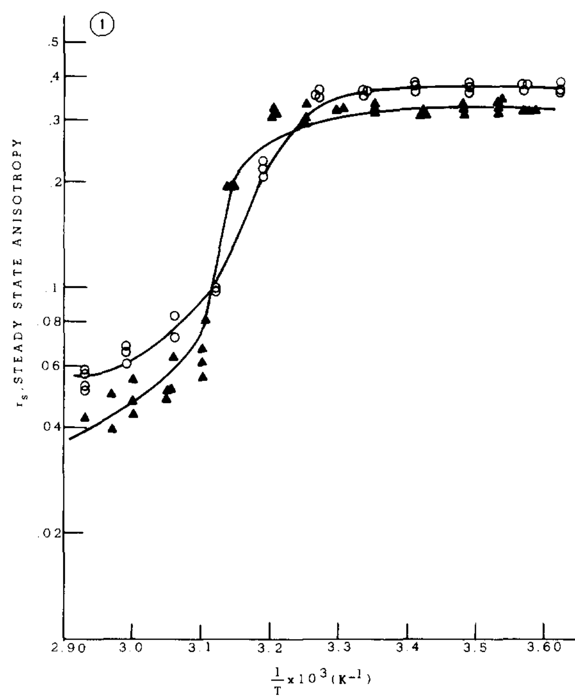
Steady-state fluorescence polarization intensity was measured with an Aminco-Bowman spectrophotofluorometer equipped with Glan-Thompson prism polarizers. Diphenylhexatriene was excited at 366 nm and fluorescence at 450 nm detected through a Wratten 2A cutoff filter for wavelengths shorter than 415 nm. Fluorescence intensities were measured with a photon counter which is suitable for the dilute systems needed to avoid light-scattering errors. Anisotropies were obtained from the intensities of emission polarized parallel and perpendicular to the polarized excitation beam by use of standard formulae including a grating correction factor [8,9].

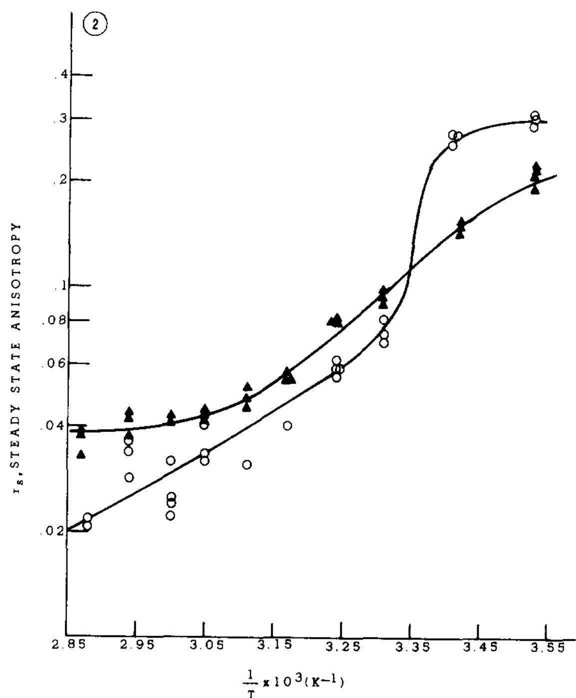
Results and Discussion

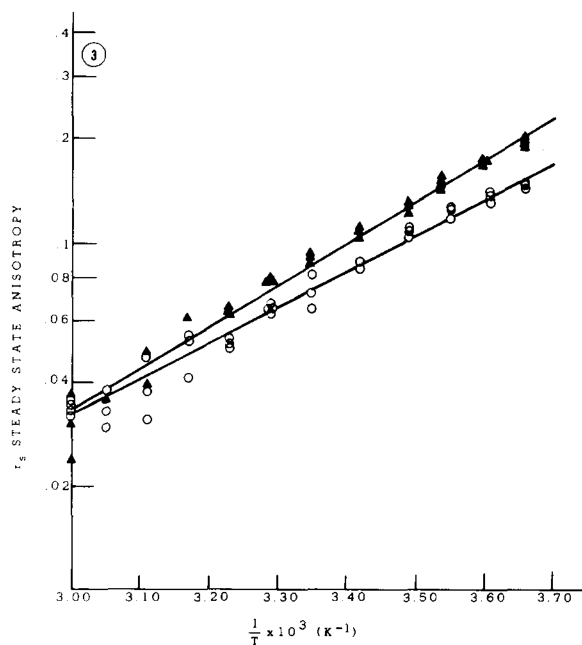
Steady-state anisotropies of fluorescence of diphenylhexatriene in the several lipid samples at various temperatures are shown graphically as Arrhenius plots in Figs. 1–7. Effects of fatty acyl unsaturation and glycerolipid structure on thermotropic behavior are evident in these data. Slope changes in the Arrhenius diagrams indicating phase transitions are apparent for the saturated and the *trans* unsaturated lipids but not for lipids with *cis* unsaturated fatty acids except nervonic (Fig. 6). Graphs for the saturated compounds 1,2- and 1,3-dipalmitoylglycerol (Fig. 1) and 1,3- and 2,3-dipalmitoylphosphatidylcholine (Fig. 7) show clearly defined narrow transition temperature intervals which include $t_{0.08}$ and t_f. The DSC-determined t_f of 63°C for 1,3-dipalmitoylglycerol is somewhat higher than the indicated transition temperature in the Arrhenius plot, however, there is some scatter in the higher temperature fluorescence polarization data. We observed similar scattering of high-temperature data in our earlier work [7]. Esters of oleic (Fig. 3) and linoleic (Fig. 4) acids show linear Arrhenius plots for r_s for the temperature interval studied which included $t_{0.08}$ in all cases and t_f in the case of 1,3-dioleoylglycerol.

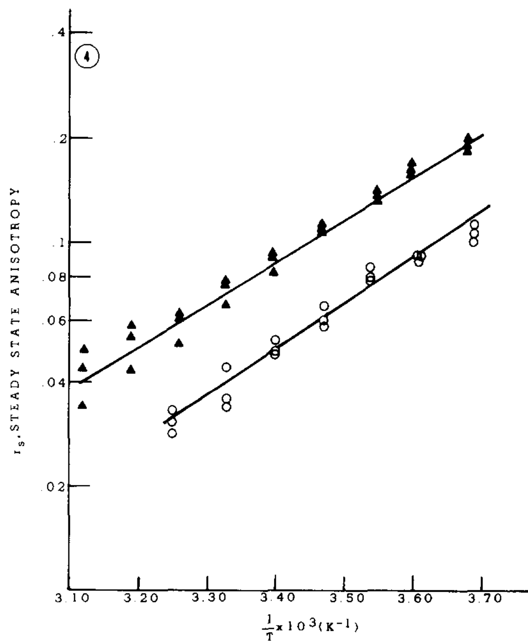
Transition temperatures for the various lipids given in Table I include $t_{0.08}$ from the fluorescence polarization data and t_f from differential scanning calorimetry. Differences between these values, $\Delta t = t_{0.08} - t_f$, are taken as indicators of residual order in melted lipids, with a large positive Δt value describing a less fluid liquid. The negative values for Δt in some instances indicate that those materials reach a highly fluid intermediate state with $S_{DPH} = 0$ without being in the liquid state. Examination of these data reveal the importance of adjacent *cis* unsaturated acyl chains in reducing fluidity. The respective Δt values of 60.9 and 13.6 for 1,2- and 1,3-dioleoylglycerol indicate substantially reduced fluidity in only the liquid 1,2 molecule where the oleoyl residues are bound to adjacent carbon atoms of the glycerol. These results support our explanation [7] for the differences in Δt values for saturated and *cis* unsaturated triacylglycerols in terms of interaction between acyl chains and the effects upon ordering caused by the kink introduced in the hydrocarbon chain by the *cis* double bond. Thus, 1,3-dilinoleoylglycerol was no less fluid than monolinoleoylglycerol. The saturated 1,2- and 1,3-dipalmitoylglycerols were equally fluid in the liquid state. Residual order after melting was also absent in the saturated phospholipids with identical Δt values for L-2,3- and 1,3-dipalmitoylphosphatidylcholine. The *trans* unsaturated compounds mono-, 1,3-di- and trielaidin show no order in the liquid state as would be expected since the conformations of the *trans* unsaturated and saturated hydrocarbon chains are similar.

Double-bond location in the acyl chains had little effect on $t_{0.08}$ in either the *cis* or the *trans* triacylglycerols (Table I), however, t_f was lowest when the *cis* double bond was located at the center of the hydrocarbon chain as in triolein (*cis* $18:1\Delta9$). Fusion temperatures were higher in both tripetroselinin (*cis* $18:1\Delta6$) and trinervonin (*cis* $24:1\Delta15$). In contrast, for the *trans* compounds, t_f









Figs. 1–7. Arrhenius plots of steady-state anisotropy of fluorescence of diphenylhexatriene.

Fig. 1. In dipalmitoylglycerol isomers. ○, 1,3-Dipalmitin; ▲, 1,2-dipalmitin.

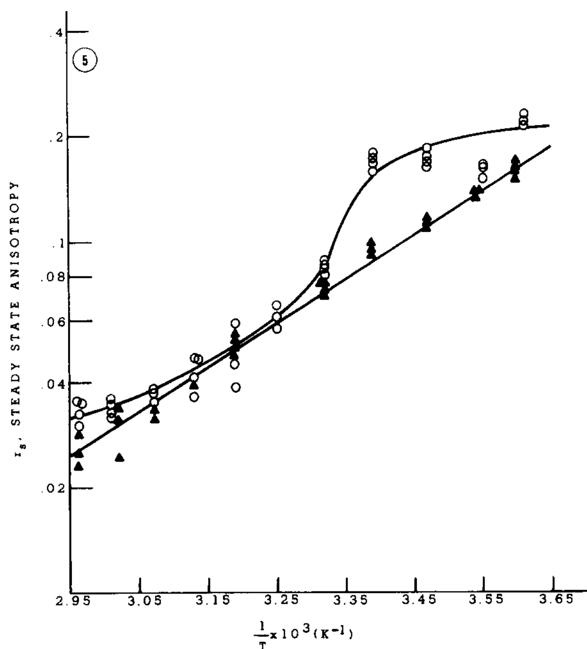
Fig. 2. In elaidoylglycerols. ○, Monoelaidin; ▲, 1,3-dielaiden.

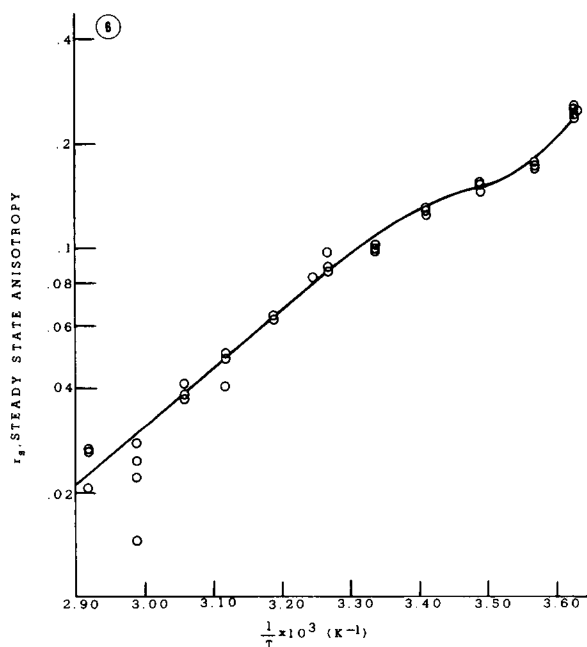
Fig. 3. In dioleoylglycerol isomers. ○, 1,2-Diolein; ▲, 1,3-diolein.

Fig. 4. In dilinoleoylglycerol isomers. ○, Monolinolein; ▲, 1,3-dilinolein.

for trielaidin (*trans* 18 : 1Δ9) was higher than t_f for tripetroselaidin (*trans* 18 : 1Δ6). Barton and Gunstone [10] reported that for *cis* unsaturated phosphatidylcholines, the transition temperature was at a minimum when the double bond was at the center of the hydrocarbon chain. Our results with the *cis* triacylglycerols are compatible with their data. Heyn [11] and Jähnig [12] showed that S_{DPH} values from diphenylhexatriene in DPPC liposomes were comparable to order parameter values from deuterium NMR corresponding to the 10–12 carbon-atom segment of the acyl chains, indicating that this may be the average location of the diphenylhexatriene molecule. The substantially lower Δt observed for trinervonin is thus reasonable since the double bond in nervonic acid is at the 15–16 carbon atom segment. The diphenylhexatriene molecule at the 10–12 carbon atom segment is thus experiencing the environment of essentially a saturated triacylglycerol molecule.

Melting temperatures for both hydrated and anhydrous samples of the lipids are given in Table II. The data are in agreement with published values where available. Multiple transitions were observed in the heating mode for 1,2-dipalmitin as had been observed [7] for several triacylglycerols. In determining Δt to indicate residual order, we





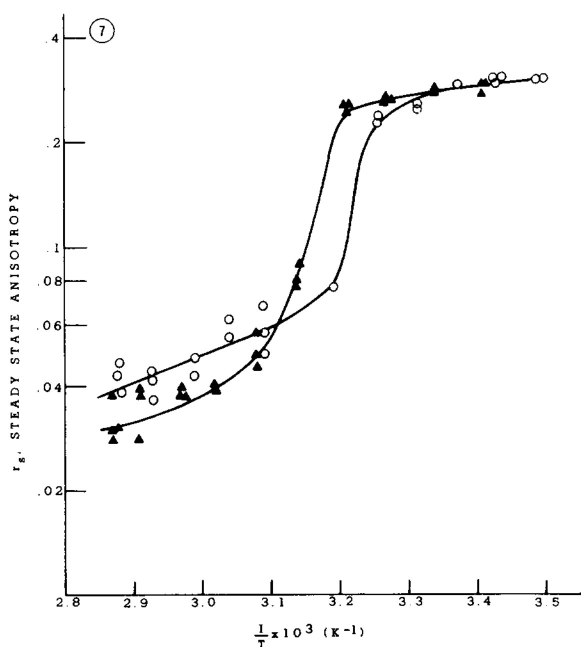


Fig. 5. In (▲) tripetroselinoyl-(*cis* 18 : 1Δ6) glycerol and (○) tripetroselaidoyl-(*trans* 18 : 1Δ6) glycerol.

Fig. 6. In trinervonoyl-(24 : 1Δ15) glycerol.

Fig. 7. In dipalmitoylphosphatidylcholines: (○) 1,3-DPPC; (▲) L-2,3-DPPC.

TABLE I

TRANSITION TEMPERATURES DETERMINED FOR LIPIDS BY DIPHENYLHEXATRIENE FLUORESCENCE POLARIZATION AND DIFFERENTIAL SCANNING CALORIMETRY

$t_{0.08}$ is the temperature for transition from a fluid state, $S = 0$, to a rigid or solid state upon cooling. t_f is the DSC-determined temperature for fusion of liquid acylglycerols upon cooling. $\Delta t = t_{0.08} - t_f$.

Lipid	$t_{0.08}$ (°C)	t_f (°C)	Δt
1,2-Dipalmitin (16:0)	48.9	50	−1.1
1,3-Dipalmitin	52.1	63	−10.9
Tripalmitin [a]	36.0	41	−5.0
1,2-Diolein (*cis* 18:1Δ9)	21.9	−39	60.9
1,3-Diolein	27.6	14	13.6
Triolein [a]	25.0	−50	75.0
1-Monolinolein (*cis* 18:2Δ9,12)	7.0	−34	41.0
1,3-Dilinolein	24.0	−6	30.0
Trilinolein [a]	16.0	< −70	> 86
1-Monoelaidin (*trans* 18:1Δ9)	28.4	21, −4	7.4
1,3-Dielaidin	33.5	43	−9.5
Trielaidin [a]	37.5	38	−0.5
Tripetroselinin (*cis* 18:1Δ6)	25.0	−12	37
Tripetroselaidin (*trans* 18:1Δ6)	28.7	15	13.7
Trinervonin (*cis* 24:1Δ15)	34.7	17	17.7
1,3-DPPC (16:0)	40.7	36	4.7
L-2,3-DPPC	45.5	41	4.5

[a] Data for these triacylglycerols cited from Ref. 7 for comparison.

TABLE II

LIPID MELTING TEMPERATURES BY DSC (°C)

Temperatures for transition from a solid or rigid state to a liquid upon heating.

Lipid	Anhydrous	Hydrated
1,2-Dipalmitin (16:0)	51, 55, 63	52, 59, 66
1,3-Dipalmitin	74	72
1,2-Diolein (*cis* 18:1Δ9)	−30	−31
1,3-Diolein	28	26
1-Monolinolein (*cis* 18:2Δ9,12)	14	−4
1,3-Dilinolein	4	3
1-Monoelaidin (*trans* 18:1Δ9)		29
1,3-Dielaidin		54
Tripetroselinin (*cis* 18:1Δ6)		−12
Tripetroselaidin (*trans* 18:1Δ6)		53
Trinervonin (*cis* 24:1Δ15)	37	36
1,3-DPPC(16:0)		39
L-2,3-DPPC		45

used t_f the fusion temperature since both the fluorescence measurements and the calorimetry were performed in the cooling mode and to avoid complexities of multiple transitions as observed on heating.

The data reported for $t_{0.08}$ and t_f thus show that positioning of acyl moieties in glycerolipids may control the fluidity of lipid dispersions or micelles. This would be true for model systems and possibly also for complexes of physiological significance such as plasma lipoproteins and/or cell membranes. Triacylglycerols are indeed significant modulators of fluidity in lipoprotein fractions from rabbit [5] and human [13] plasma. Our findings that adjacent positioning of *cis* unsaturated fatty acids induces ordering in liquid di- and triacylglycerols may provide mechanisms for the reported high levels of atherogenicity in certain dietary lipids. Kritchevsky and co-workers reported in a series of papers [14–16] that certain peanut oils are more atherogenic than others and that randomization of the oils reduced atherogenicity [15], thus relating atherogenicity to glycerolipid structure. They posed a question as to whether the differences in the peanut oils are effective at the absorption level or at the lipoprotein structure level [15]. High-density lipoproteins (HDL) are intestinally synthesized [17], hence their properties may relate to absorbed lipid structures. Ingested triacylglycerols are hydrolyzed in the intestinal lumen yielding free fatty acids and 2-monoacylglycerols, and these species are absorbed into intestinal cells wherein they are resynthesized into triacylglycerols but not necessarily of the same structure as the dietary triacylglycerols. Mattson et al. [18] did find that triacylglycerol structure affects absorption, however, these effects relate to the 2-monoester formed in the intestine. Manganaro et al. [19] reported on the molecular species of triacylglycerols in various peanut oils and demonstrated that atherogenicity is related to the level of linoleate in the *sn*-2 position of the acylglycerol. We suggest the following hypothesis for the role of peanut oil in atherogenesis. Intestinal lipolysis of the 'atherogenic peanut oil' yields more 2-monolinoleoylglycerol which is absorbed and combined with free fatty acids in intestinal cells for synthesis of triacylglycerols with inevitably more adjacent *cis* unsaturated fatty acyl moie-

ties either as *sn*-1,2 or *sn*-2,3 esters. These triacylglycerols which will form more ordered structures in the liquid state may be incorporated into HDL formed in the intestine. The less-fluid HDL may then be less capable of mobilizing and transporting tissue cholesterol. This phenomenon can result in atherogenesis as the function of HDL in preventing atherosclerosis which involves mobilizing and transporting peripheral tissue cholesterol [20,21] will be inhibited. If the absorbed 2-monolinoleoylglycerol is esterified with saturated fatty acids in the *sn*-1 and 3 positions, the kinked linoleoyl residue may also reduce freedom of rotation of the saturated residues. Seelig and Seelig [22] demonstrated oleoyl induction of order in an adjacent palmitoyl residue in 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine bilayers. Clearly, further research is needed to understand and describe the effects of isomeric acylglycerols on lipoprotein lipid structure and lipoprotein physical properties.

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