bits were bled. Precipitin patterns were observed in double diffusion agarose plate tests with the rabbit melanoma TAA antiserum for testing against melanoma TAA, neuroblastoma antigens, colon cancer antigens, fetal skin antigens, other less specific melanoma-associated antigens (*4*), and purified albumin. A specific precipitin band formed with the melanoma TAA, but not with the other antigens tested. A nonintersecting, different band formed with purified albumin. The U.S.S.R. melanoma carbohydrate extract was then compared with melanoma TAA with the antiserum for analysis of common precipitin patterns. As shown in Fig. 3, the melanoma TAA present in the U.S.S.R. extract (70 μg) was serologically identical to the U.S. melanoma TAA (10 μg). A slightly skewed pattern developed since a stronger concentration of the U.S.S.R. antigens was used. The additional band formed was similar to that for albumin. When antiserums were absorbed against purified albumin this band disappeared.

In conclusion, a very similar major specific TAA was isolated from melanoma tissue in both countries. This glycolipoprotein produces a specific cell-mediated immune response in melanoma patients of both places. This assures us that a continued comparison of ongoing studies of the immune responses of melanoma patients carried out in both countries for diagnostic, monitoring, or therapeutic purposes will be possible.

V. V. GORODILOVA
*Hertsen Scientific Research Oncology Institute, Moscow, U.S.S.R.*

A. HOLLINSHEAD
*George Washington University Medical Center, Washington, D.C. 20037*

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## Thermal Transitions in Human Plasma Low Density Lipoproteins

Abstract. *Thermal analysis of human plasma low density lipoproteins reveals a broad reversible transition encompassing body temperature. The calorimetric and x-ray scattering data identify this transition as a cooperative, liquid-crystalline to liquid phase change involving the cholesterol esters in the lipoprotein. This behavior requires the presence of a region rich in cholesterol ester within the lipoprotein.*

Although low density lipoprotein (LDL) is the major cholesterol-carrying lipoprotein in human serum and has been implicated in the etiology of atherosclerosis, its structure and the intermolecular forces required to maintain its individually insoluble lipid and protein moieties as a soluble particle have not been well defined. Essential to such an understanding is a knowledge of the lipid-lipid and lipid-protein interactions within the particle. The interactions between different lipid classes, such as phospholipids, free and esterified cholesterol, and triglycerides have been studied by, for example, differential scanning calorimetry, polarizing light microscopy, x-ray diffraction, and various spectroscopic methods (*1–3*).

Thermal transitions, reflecting either polymorphic or liquid crystalline transitions, have been observed with phospholipids, cholesterol esters, and triglycerides, and in addition, calorimetric methods have revealed phase transitions associated with the lipid and protein components of cell membranes (*4*). In this report, we describe a reversible transition due to a cooperative order-disorder phase change involving the cholesterol esters within intact LDL, and an irreversible transition associated with lipoprotein denaturation.

We isolated LDL from plasma of normal human donors by preparative salt density ultracentrifugation (*5*), and concentrated it to about 200 mg of LDL per milliliter (*6*). The protein concentration was determined by the method of Lowry *et al.* (*7*), and the total LDL concentration was estimated from the protein content and by dry weight determination. Lipid composition was analyzed by quantitative thin-layer chromatography (*8*) of the chloroform-methanol-extracted lipids (*9*). Individual lipid classes were isolated by preparative thin-layer chromatography.

Six preparations of LDL were studied with a Perkin-Elmer DSC-2 differential scanning calorimeter at a full-scale sensitivity of 0.1 to 0.2 mcal/second with heating and cooling rates of 10°C per minute. Differential scanning calorimetry curves of a typical LDL preparation are shown in Fig. 1. Concentrated native LDL shows a reversible transition between 20° and 45°C. The peak of the endothermic transition occurs, depending on the sample (*10*), between 29° and 38°C, and the enthalpy ($\Delta H$) of this transition is 0.4 ± 0.1 cal per gram of LDL lipid (mean ± standard deviation). The transition is unchanged on repeated heating between 0° and 45° (Fig. 1a), and it is not significantly affected by cooling the LDL samples to −60°C and reheating (Fig. 1, b and d). On cooling, little supercooling, if any, is observed (Fig. 1c). Finally, the transition is unaffected over a 30-fold change in LDL concentration (13 to 400 mg/ml) or by NaBr concentrations up to 2*M* NaBr.

An irreversible transition ($\Delta H$ = 0.9 ± 0.2 cal per gram of LDL protein), seen only on initial heating, occurs between 70° and 90°C (Fig. 1d) and corresponds to irreversible changes in the optical properties of LDL observed by polarizing light microscopy (*11*). We have defined these irreversible changes as "lipoprotein denaturation." Following denaturation and cooling to −60°C, a transition is present at 5° to 45°C (Fig. 1e) with an enthalpy at least five times greater than that of the initial reversible transition (Fig. 1, a to d).

X-ray scattering and diffraction from LDL and its cholesterol ester extracts (Fig. 2) were recorded at temperatures above (45°C) and below (10°C) the low temper-

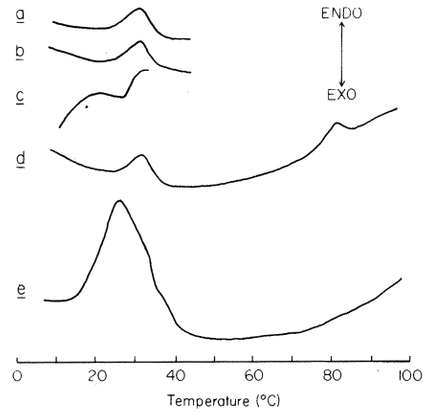


Fig. 1. Differential scanning calorimetry curves of a single LDL sample. Approximately 10 μl of concentrated solution containing 2.05 mg of LDL was placed in a sealed sample pan and an equivalent amount of the solvent 0.19*M* NaCl in the reference pan. Samples were studied between 0° and 45°C and between 0° and 100°C, with and without prior cooling to −60°C. Samples cooled to −60°C were heated at 10°C per minute and held at 1°C until the ice-liquid water transition was complete, and then the heating run was continued. (a) Initial heating curve from 0° to 45°C; (b) heating curve 0° to 45°C after cooling to −60°C; (c) cooling curve from 45° to 0°C; (d) heating curve from 0° to 100°C after cooling to −60°C; (e) heating curve from 0° to 100°C after heating to 100°C and cooling to −60°C.

ature transition observed by calorimetry. At 10°C, the low-angle x-ray scattering maxima from the quasi-spherical LDL particles are similar to those previously reported by Mateu *et al.* (*12*). A strong diffraction fringe corresponding to a Bragg spacing of 36 Å is present (Fig. 2a). This diffraction line is not observed at 45°C (Fig. 2b) but reappears on cooling. To prove that transitions observed by calorimetry correlated with the x-ray data, lipoproteins were studied at small temperature intervals from below to above the calorimetric transition. A strong 36 Å maximum was present at a temperature just below the transition, decreased in intensity in the range of the transition, and was not observed just above the transition.

After denaturation, at 10°C, all of the low-angle x-ray scattering maxima have disappeared with the exception of that at 36 Å (Fig. 2c). Again, this maximum is not observed at 45°C (Fig. 2d) but reappears on cooling. At 25°C the cholesterol ester fraction isolated from LDL exists in a uniaxial smectic liquid crystalline state as demonstrated by polarizing light microscopy and shows a similar x-ray diffraction line at 36 Å (Fig. 2e) (*13*). The separated cholesterol ester fraction from each LDL melts from the smectic to the isotropic state, over a temperature range nearly identical to the transition range of the parent native LDL. In the isotropic state, no well-defined spacing at 36 Å is present (Fig. 2f). Similar temperature dependence of the low angle x-ray spacing at 36 Å is shown by cholesteryl oleate, cholesteryl linoleate (*3, 14*), and mixed liquid crystalline solutions of cholesteryl oleate and linoleate (see Fig. 2, g and h).

Analysis of the lipid composition of LDL shows that cholesterol esters constitute 56 ± 3 percent, by weight, of the total lipids, with values of 12, 28, and 4 percent for free cholesterol, phospholipid, and triglyceride, respectively. The apoprotein contributes 22 to 24 percent of the particle weight. If the enthalpy of the initial transition (0.4 cal per gram of total LDL lipid) is expressed in terms of the cholesterol ester present in LDL, the enthalpy corresponds to 0.7 ± 0.1 cal per gram of cholesterol ester. The enthalpy for the cholesterol esters extracted from LDL is 1.0 ± 0.1 cal/g. These values are in good agreement with enthalpy values for liquid crystalline to liquid transitions of cholesteryl oleate or cholesteryl linoleate ($\Delta H$ = 0.8 to 1.1 cal/g) (*15*) and for mixtures of these two esters ($\Delta H$ = 1.0 cal/g) (*16*). The lower enthalpy observed in LDL may reflect the constraints placed on the cholesterol ester molecules in the LDL particle, such as those due to structural limitations arising from the size of the lipoprotein particle and interactions of some of the cholesterol ester molecules with other molecular constituents of the lipoprotein. After lipoprotein denaturation, the behavior of the cholesterol esters is no longer influenced by the structure of the particle, and the separated cholesterol esters are now able to crystallize when cooled sufficiently (*17*). The melting of these crystals accounts for the marked increase in enthalpy shown in Fig. 1e.

Thus, intact LDL gives an x-ray diffraction fringe at 36 Å whose thermal behavior parallels that of (i) the smectic liquid crystalline phase of cholesterol esters in denatured LDL, (ii) cholesterol esters isolated from LDL, and (iii) mixtures of cholesterol esters. Finally, scanning calorimetric studies of the other individual lipids isolated from LDL (phospholipids, cholesterol, and triglycerides), as well as the isolated lipid-free apoprotein, showed no transitions in the range 20° to 60°C. These findings indicate that the endothermic transition between 20° and 45°C in intact LDL is due to a smectic liquid crystal to liquid phase change associated with the cholesterol esters present in the LDL particle.

Our results are difficult to reconcile with the spherical lipid bilayer model of LDL proposed by Mateu *et al.* (*12*), in which the cholesterol ester molecules are incorporated into a phospholipid bilayer 40 Å thick. This could not result in either layered arrangements of cholesterol ester molecules or cooperative phase transitions of the magnitude observed. The minimum domain size of the cholesterol ester cooperative unit estimated from the van't Hoff and calorimetric enthalpies of the transition in intact LDL (*18*) is about 50 to 100 molecules. In addition, preliminary analysis of the width of the 36 Å diffraction fringe (*19*) suggests a repeating layered arrangement of cholesterol ester molecules in LDL with a thickness of about 150 Å. These observations necessitate the existence of a region within the LDL particle,

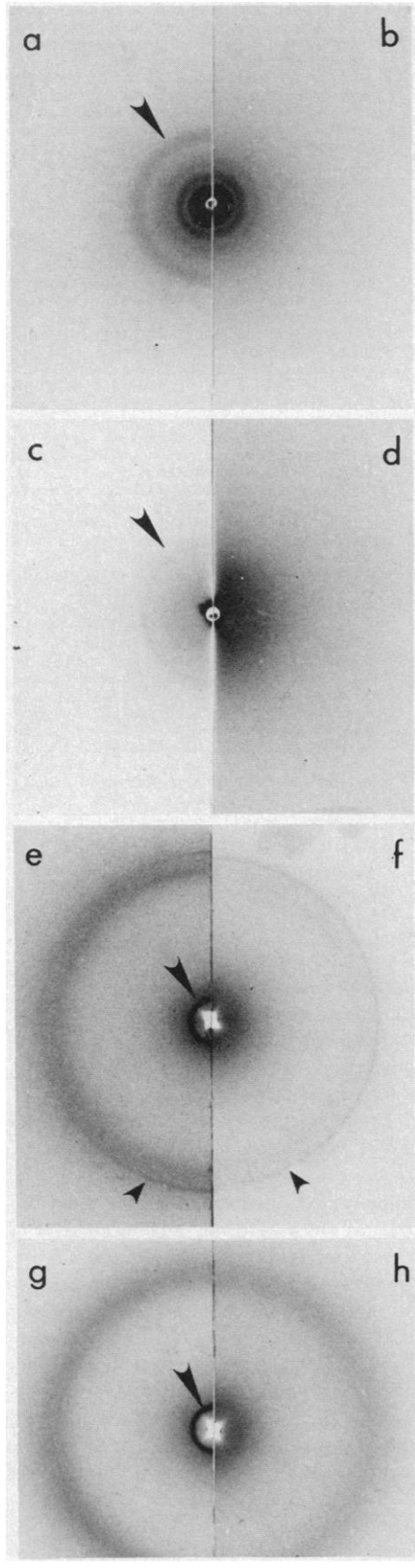


Fig. 2. X-ray scattering/diffraction patterns of LDL and cholesterol esters below and above their respective transition temperatures (see text). Specimens were sealed in either Lindemann glass tubes (1 mm diameter) or between two Mylar windows and placed in a variable-temperature sample holder. X-ray scattering/diffraction patterns were recorded with focusing cameras with either Elliott toroidal mirror or Franks' double mirror optics, utilizing nickel filtered $CuK_\alpha$ radiation from an Elliott GX-6 rotating anode generator. LDL solution at 10°C (a) and 45°C (b); LDL solution (after denaturation by heating to 100°C) at 10°C (c) and 45°C (d); cholesterol esters extracted from LDL at 25°C (e) and 45°C (f); mixture of cholesteryl oleate and linoleate (6 : 4 weight ratio) at 30°C (g) and 45°C (h). In all cases, the fringe representing the 36-Å spacing (arrow) is present below and absent above the transition. A diffuse maximum representing a Bragg spacing of 4.8 Å is present on films recorded with the toroidal optical system, (e) to (h). [The small arrows on (e) and (f) indicate sharp diffraction lines from the small Teflon O-ring sample holder used when only small amounts of sample were available.]

rich in cholesterol ester, and large enough to permit a layered liquid crystal arrangement capable of a cooperative melting behavior.

R. J. DECKELBAUM
G. G. SHIPLEY, D. M. SMALL
*Divisions of Gastroenterology and Biophysics, Department of Medicine, Boston University School of Medicine, Boston, Massachusetts 02118*
R. S. LEES, P. K. GEORGE
*Arteriosclerosis Center, Massachusetts Institute of Technology, Cambridge 02139*

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6. LDL was concentrated to 150 to 250 mg/ml by dialysis against dextran of high molecular weight (molecular weight 252,000; Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N.Y.), or by vacuum dialysis in a Selectron collodion bag glass apparatus (Schleicher and Schuell, Inc., Keene, N.H.).
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17. We have demonstrated the presence of cholesterol ester crystals at 10° to 20°C, by polarizing light microscopy and x-ray diffraction in both the denatured LDL sample and the cholesterol ester extract when cooled to −60°C and reheated.
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## Binaural Beats at High Frequencies

Abstract. *Binaural beats have long been believed to be audible only at low frequencies, but an interaction reminiscent of a binaural beat can sometimes be heard when different two-tone complexes of high frequency are presented to the two ears. The primary requirement is that the frequency separation in the complex at one ear be slightly different from that in the other—that is, that there be a small interaural difference in the envelope periodicities. This finding is in accord with other recent demonstrations that the auditory system is not deaf to interaural time differences at high frequencies.*

For decades it has been known that the auditory system is provided with two binaural cues for localizing sound sources—interaural time differences and interaural intensity differences—and on the basis of certain physical and psychophysical facts it has been commonly asserted that the two cues are functional in different spectral regions. Interaural intensity differences have been thought to be of value only for high frequencies and interaural time differences only for low frequencies. In part, this belief (sometimes expressed as the duplex theory of sound localization) stemmed from psychophysical research using sinusoidal signals as the waveforms to be localized. For these simplest of waveforms, there is no argument—the auditory system is insensitive to interaural time differences above about 1200 to 1500 hertz (*1*)—but many psychoacousticians applied duplex theory to other listening situations as well, and this has recently been shown to have been an error. Recent research (*2, 3*) shows that more complex waveforms provide the system with a processable time cue in addition to the cycle-by-cycle time differences available with sinusoids. That is, a complex waveform that is time-delayed to one ear provides the auditory system with interaural time differences in the envelope of the waveform, and it is now clear that the auditory system can lateralize (*4*) just as accurately at high frequencies working on this cue as it can at low frequencies working on cycle-by-cycle time differences—only a few microseconds are required for excellent performance.

The realization that the auditory system is not deaf to interaural time differences at high frequencies led us to wonder if there might be any other time-based phenomena that were known not to occur with high-frequency tones, but that might be detectable using more complex waveforms. Among the first to come to mind was binaural beats. It has long been known that if one low-frequency tone is presented to one ear only and a second tone, slightly different in frequency, to the other ear only, a beat will be heard whose rate is equal to the difference in frequency between the tones. Since the two waveforms are not being mixed acoustically, this beat must be the result of an interaction somewhere in the auditory nervous system, and the system must have preserved the "fine structure" (the cycle-by-cycle periodicities) of the two waveforms for this interaction to have occurred. The fact that a binaural beat cannot be heard if the two tones exceed about 1000 hertz (*5*) implies that the auditory nervous system preserves cycle-by-cycle periodicities with diminishing accuracy beyond this point, and this view is reinforced by the fact that sound localization on the basis of cycle-by-cycle time differences also begins to deteriorate beyond this frequency region (*1*). But while all this is true for tonal stimuli, might not an interaction similar to binaural beats be possible for the complex high-frequency waveforms for which time-based lateralization is now recognized to be possible?

We found that it is possible to hear a binaural beat at high frequencies by using complex waveforms whose envelope periodicities are slightly different at the two ears. For example, if 3000 hertz is presented to both ears, 3100 hertz only to the left, and 3101 hertz only to the right, then the envelope periodicities in the two ears differ by 1 hertz, and a faint, one-per-second beat is detectable. To our knowledge, this binaural beat at high frequencies has not been previously reported.

We have done several things to convince ourselves that the effect does involve an interaction between the envelopes in the two ears. One of our first concerns was with combination tones or distortion products (*6*), for if low-frequency products were being generated by nonlinearity in the auditory periphery, it could well have been