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KINETICS OF THERMAL TRANSITIONS IN HUMAN SERUM LOW DENSITY LIPOPROTEINS (LDL) AND NEUTRAL LIPIDS. A DYNAMIC SMALL-ANGLE X-RAY SCATTERING STUDY

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ABSTRACT

The time course of the temperature induced structural transitions in the human serum low density lipoproteins (LDL) and in a cholesteryl linoleate-triglyceride mixture was followed by small-angle X-ray scattering using a linear position-sensitive detector. The kinetics of the near body temperature transition was very simliar in both systems. However, at low temperatures the structural changes observed in LDL showed an entirely different kinetics as compared to the neutral lipid mixture.

CINETICA DE TRANSICIONES TERMICAS EN LIPOPROTEINAS DE BAJA DENSIDAD DE SUERO HUMANO (LDL) Y EN LIPIDOS NEUTROS. ESTUDIO POR DIFRACTOMETRIA DINAMICA DE RAYOS X (DDRX) A PEQUEÑO ANGULO

RESUMEN

El curso temporal de las transiciones estructurales inducidas por temperatura en lipoproteínas de baja densidad de suero humano (LDL) y en una mezcla de linoleato de colesterol-triglicéridos, fue determinada por difusión central de rayos X usando un detector lineal sensible a posición. La cinética de las transiciones observadas a temperaturas cercanas a la corporal fue muy similar en LDL y en la mezcla de lípidos neutros; mientras que, a bajas temperaturas la cinética de transición fue completamente diferente en ambos sistemas.

INTRODUCTION

During the last few years the linear position-sensitive detectors[1] have been successfully employed for the investigation of the kinetical aspects of structural modifications in biological systems as induced by temperature,[2] chemical compounds,[3] drying and rehydration,[4] etc. One interesting problem to be studied by this technique is related to the various thermal transitions observed in human serum low density lipoproteins (LDL) and in neutral lipids derived from these particles.

LDL produces a characteristic X-ray scattering pattern[5] consisting of 5 fairly sharp low-angle maxima and a band centered at $\mathcal{S}=(36\ \text{Å})^{-1}$ whose intensity is temperature dependent.[6] Indeed, at 2°C the intensity of this band is very strong, progressively decreasing as the temperature increases and finally completely disappearing near body temperature.[7,8,9] This thermal behaviour was interpreted as due to a liquid crystal→isotropic liquid transition of the neutral lipids within the particle.[8,9] Moreover, we have recently shown[4,10] that the disappearence of the $(36\ \text{Å})^{-1}$ band can also be induced at temperatures below 0°C.

The X-ray diffraction pattern of the smectic phase of neutral lipids from LDL shows a sharp and strong single reflection also centered at around $\mathcal{S}=(36\ \text{Å})^{-1}$ which on melting to the liquid isotropic phase transforms to a very broad band centered at around $\mathcal{S}=(30\ \text{Å})^{-1}$. In contrast, the crystalline phase shows several sharp reflections which extend up to higher angles.[11]

In this work we report for the first time a fast small-angle X-ray scattering study of the thermally induced structural transitions in LDL and in a lipid mixture which contains the major neutral components of the lipoproteins (cholesteryl linoleate and trygliceride). Our purpose was to determine the kinetics of the various transitions observed in the pure neutral lipid system as compared to those described for LDL.

METHODS

The samples

LDL of d=1.019−1.063 was prepared in pure form from fresh human serum from male donors as previously decribed.[4,7] After purification the LDL was concentrated by centrifugation at 45.000 rpm in a swinging bucket rotor (Beckman SW-65) during 48 h in a buffer containing: 150 mM NaCl, 0.5 mM Na_2—EDTA, 5 mM Tris—HCl (ph=7.4). The sample was recovered as an orange-colored transparent pellet at the bottom of the tubes.

The neutral lipid sample was prepared by addying 80 mg of cholesteryl linoleate (purchased from SIGMA CO., and used without further purification) to 20 mg of a mixture of more than 98% pure triglycerides (fatty acid composition: C16:0,10%; C18:0,4%; C18:1,41%; C18:2,45%). The lipids were solubilized in chloroform and the solvent was evaporated at 50°C, first under a nitrogen stream and then under vacuum. An aliquot of this neutral lipid sample was used for the X-ray experiment.

X-ray techniques

CuK_α radiation was obtained from an Elliott GX6 rotating-anode X-ray generator with a nickel coated optical flat, operating with linear collimation. The X-ray patterns were recorded with a linear position-sensitive detector[1] and stored in an on-line computer. In some experiments two position sensitive detectors were used for simultaneous recording of both the low and the high angle regions of the reciprocal space. The data acquisition intervals were 15 seconds for LDL and either 2, 10 or 15 seconds for the neutral lipid sample. The counting error per channel was estimated to be of less thant 10% in all the cases. The computer-stored X-ray patterns were displayed on a cathode ray tube and photographed onto 35 mm film.

Experimental arrangement

Figure 1 shows the experimental set-up employed for the kinetic measurements. The sample 1 mm thick was

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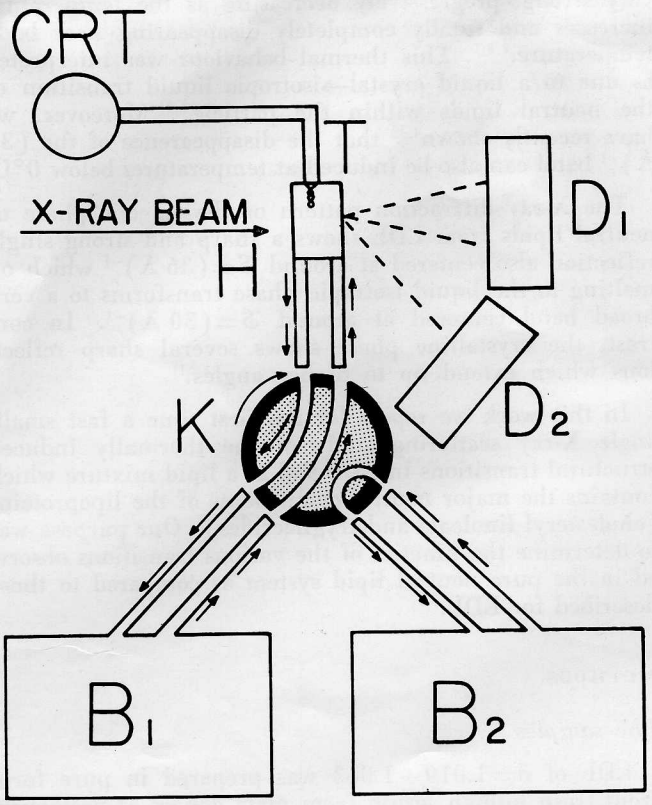

Figure 1: Experimental arrangement - The sample is located within a holder (H) connected to either of two constant temperature bath circulators (B1, B2) which are kept at the initial and final temperature of the experiment. The temperature jump is applied by rapidly turning a valve key (K) which switches the circulation from one bath to the other. A copper-constant thermocouple (T) placed within the sample and connected to an storage oscilloscope (CRT) permits to determine the temperature changes instantaneously. D1 and D2 are one-dimensional position-sensitive detectors for the simultaneous recording of the low and high angle scattering regions. See text for details.

mounted in a tight holder (H) between two thin beryllium plates in order to assure a good thermal contact. The holder was connected to a constant temperature circulator bath (B1) via a three-double way valve key (K) which permits to flux the liquid from the B1 bath through the holder and the simultaneous circulation of the liquid of a similar bath (B2) through the valve. B1 and B2 are mantained respectively at the initial and final temperatures desired. Turning the valve by 120° allows to rapidly connect the holder to either of the two baths, this manipulation takes less than 1 second. A copper-constant thermocouple (T) placed within the sample was connected to a storage oscilloscope (CRT). The voltage deflection observed on the CRT screen was photographed with a polaroid camera. The time dependence of the trace deflection was measured in the photographs. This set up allowed us the instantaneous evaluation of the temperature of the sample with an accuracy of ± 0.5°C. The time constant of the applied thermal steps was of less than 5 seconds.

RESULTS

X-ray patterns at thermal equilibrium

Figure 2a shows a 15 minutes counting X-ray scattering pattern of LDL recorded at 2°C. It consists of 5 fairly sharp low angle maxima and a band centered at $\mathcal{S}=(36\ \text{Å})^{-1}$. As stated above, the intensity of the band was found to be temperature dependent.[7,8,9] Figure 2b shows the pattern recorded at 37°C which is similar to that shown at 2°C with the exception of the absence of the $(36\ \text{Å})^{-1}$ band. Figure 2c shows the pattern from the same sample recorded at very low temperatures (−10°C), in which the case the $(36\ \text{Å})^{-1}$ band is also absent. At low temperature the intensity of the band is replaced by a residual scattering centered at $\mathcal{S}=(45\ \text{Å})^{-1}$ (not shown in the figure) whose structural significance is being investigated at present (L. Mateu, work in progress).

The X-ray diffraction pattern of the smectic phase of cholesteryl linoleate-triglyceride mixture recorded at 3°C shows a sharp reflection centered at around $\mathcal{S}=(36\ \text{Å})^{-1}$ (Fig. 3a). At 32°C the smectic phase transforms to the liquid isotropic phase characterized by a very broad band

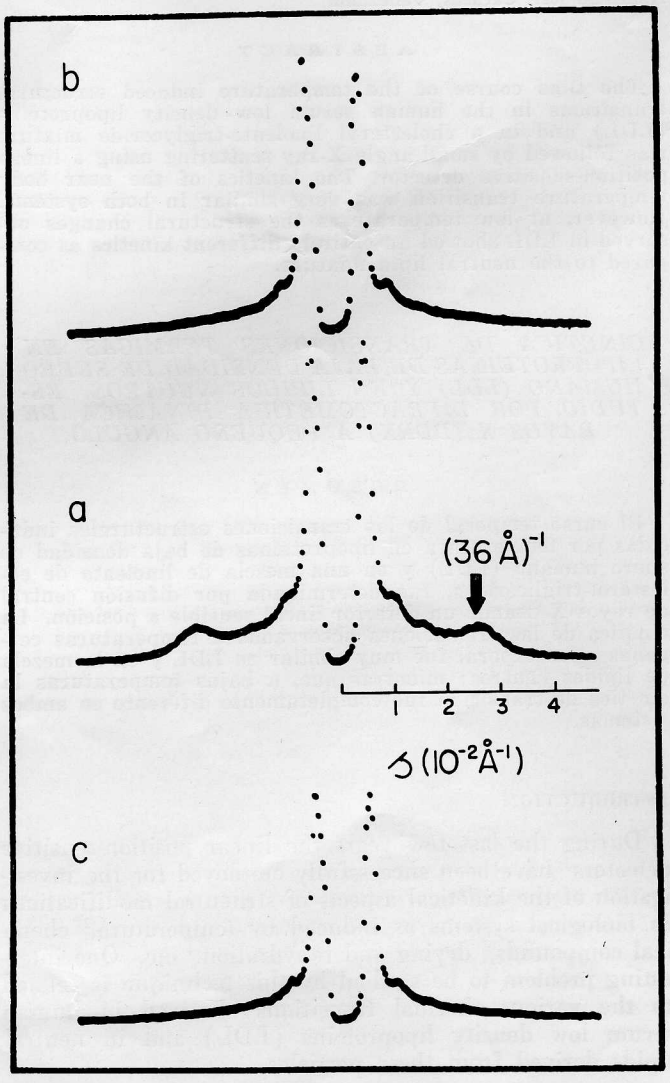

Figure 2: Small-angle X-ray scattering patterns accumulated during 15 minutes from an LDL sample in equilibrium at different temperatures. a, Spectrum recorded at 2°C. The $(36\ \text{Å})^{-1}$ band is pointed by an arrow. b, c, Spectra recorded at 37 and −10°C respectively. Notice the absence of the $(36\ \text{Å})^{-1}$ band in both registers. $\sigma=\frac{(2\sin\theta)}{\lambda}$ (2 θ is the scattering angle, λ=1.54 Å is the wavelenght).

centered at around $\mathcal{S}=(30\ \text{Å})^{-1}$ (Fig. 3b). The crystalline phase recorded at $-3°C$ shows a strong reflection centered at around $\mathcal{S}=(18\ \text{Å})^{-1}$ (Fig. 3c) and several additional intensities which extend up to higher angles ($\mathcal{S}<(3\ \text{Å})^{-1}$) (not shown in the figure).

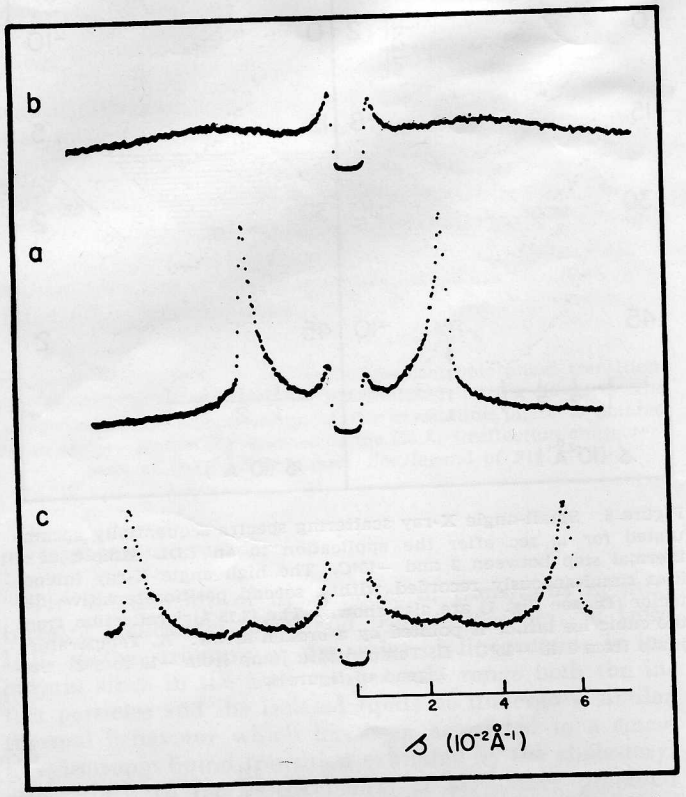

Figure 3: Small-angle X-ray diffraction patterns accumulated during 5 minutes from a cholesteryl linoleate-triglyceride sample (see Methods) in equilibrium at different temperatures. - a, Spectrum recorded at 3°C showing the sharp and strong (36 Å)$^{-1}$ reflection characteristic of the smectic phase. b, Spectrum recorded at 42°C showing the (30 Å)$^{-1}$ broad band characteristic of the isotropic phase. c, Spectrum recorded at −3°C. The pattern is mainly composed by a sharp and strong reflection centered at (18 Å)$^{-1}$. Several much weaker reflections are present at higher angles (not shown in the figure).

In agreement with previous observations[8,11,12] the liquid isotropic → cholesteric transition was not observed since the sample contains large amounts of triglycerides.

Kinetical studies

In order to determine the time course of the transitions whose equilibrium states were shown in figures 2 and 3, temperature steps were applied to the samples and X-ray spectra were sequentially recorded as indicated in Methods. In determining these phenomena different kinetics were distinguished which we are going to describe below.

a) *Fast transitions*

Fast transitions were observed in both systems at near body temperature. Figure 4A shows several 15 sec counting spectra sequentially recorded after the application to an LDL sample of a thermal step between 2 and 38°C. The spectrum at "0" sec was recorded at 2°C as a control "at equilibrium". The thermal step was applied simultaneously with the recording of a new spectrum, at the end of this accumulation the temperature was 36°C (Fig. 4A "15" sec). At this time, the (36 Å)$^{-1}$ band was already absent. As shown in figure 4B the reverse transition was similar. In both directions it was completed in less than 15 seconds as measured by the presence or the absence of the (36 Å)$^{-1}$ band and it could not be followed with our detection method.

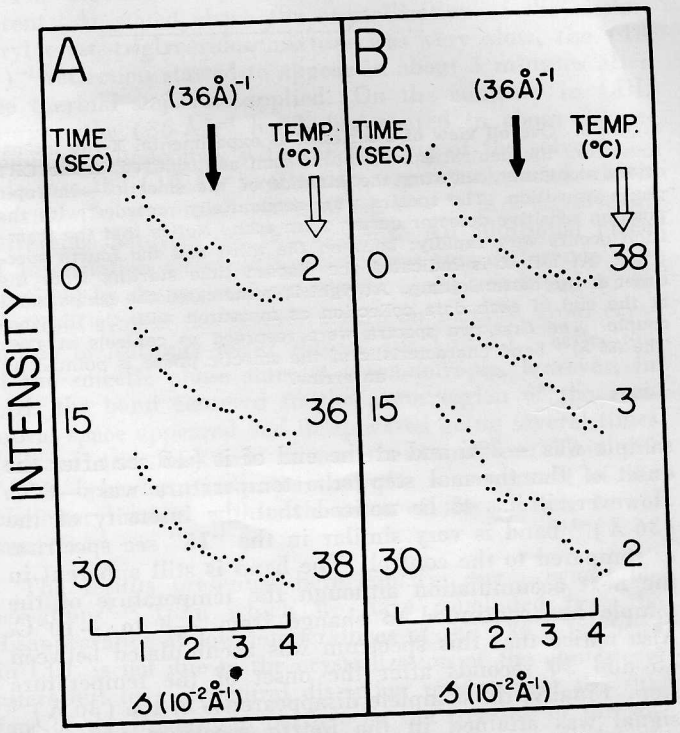

Figure 4: Small-angle X-ray scattering spectra sequentially accumulated for 15 sec after the application to an LDL sample of a thermal step between 2 and 38°C. The elapsed time (sec) starting from the onset of the thermal step is indicated for each spectrum (at left). The temperature measured at the end of each data collection is also shown (at right). Full arrows point to the (36 Å)$^{-1}$ band. Empty arrows indicate the direction of the thermal step. A, temperature jump from 2 to 38°C. B, temperature jump from 38 to 2°C.

It is interesting to note that the smectic ↔ isotropic transition in the neutral lipid system also takes place very rapidly. Figure 5 shows an overall view of 16 consecutive 2 sec counting spectra sequentially recorded from the neutral lipid sample after the application of the thermal step (see legend of Fig. 5). Since the lipid sample scattered more strongly than the LDL sample the (36 Å)$^{-1}$ band characteristic of the smectic phase is very well observed in this short time. The smectic ↔ isotropic transition took place in less than 4 seconds (see Fig. 5) and it could not either be followed.

b) *Slow transitions*

The fast above phenomena contrasted with the slow structural modifications at lower temperatures. Figure 6A shows several 15 sec counting spectra sequentially recorded after the LDL sample was rapidly cooled from 2 to −10°C. The "0" sec spectrum was recorded "at equilibrium" at 2°C and clearly shows the (36 Å)$^{-1}$ band. At the end of this accumulation the thermal step was applied and simultaneously the recording of the second spectrum ("15" sec Fig. 6A) was initiated. Within the first 7 sec of this accumulation the temperature of the

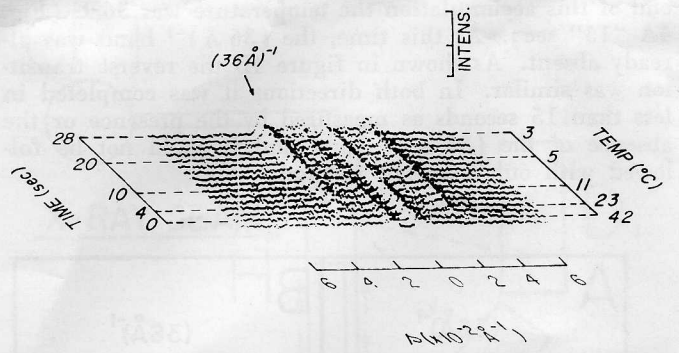

Figure 5: Overall view of a series of 16 experimental X-ray intensities from the neutral lipid mixture such as displayed in the CRT of the computer, showing the kinetics of the smectic⟷isotropic phase transition. The spectra were sequentially recorded with the position sensitive detector during 2 sec each. Notice that the transition occurs very rapidly: between the third and the fourth spectrum. At left it is indicated the elapsed time starting from the onset of the thermal jump. At right it is indicated the temperature at the end of each data collection as measured with the thermocouple. The first two spectra were recorded as controls at 42°C. The (36 Å)$^{-1}$ band characteristic of the smectic phase is pointed by an arrow.

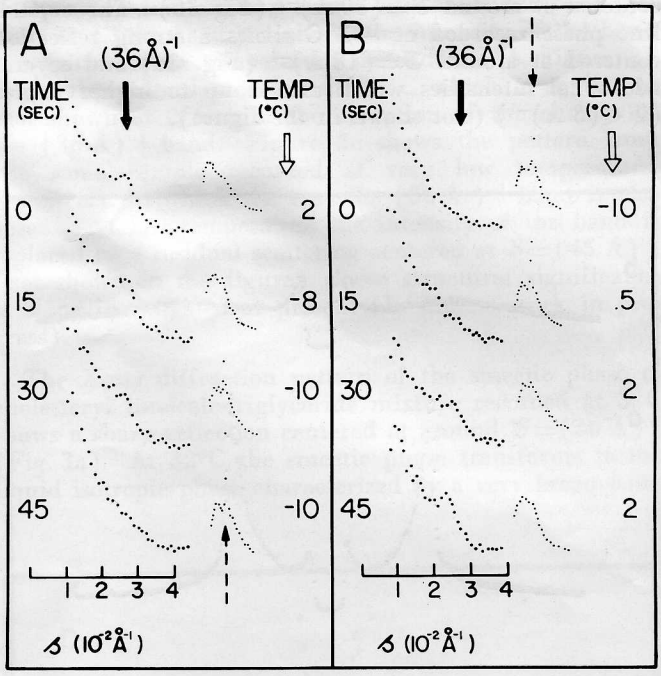

Figure 6: Small-angle X-ray scattering spectra sequentially accumulated for 15 sec after the application to an LDL sample of a thermal step between 2 and −10°C. The high angle X-ray intensities simultaneously recorded with a second position-sensitive detector (D_2, see Fig. 1) are also shown. The (3.75 Å)$^{-1}$ reflection from the cubic ice lattice is pointed by a broken arrow. A, Temperature jump from 2 to −10°C. B, Temperature jump from −10 to 2°C. See legend of figure 4.

sample was −3°C and at the end of it (15 sec after the onset of the thermal step) the temperature was −8°C. However, it has to be noticed that the intensity of the (36 Å)$^{-1}$ band is very similar in the "15" sec spectrum as compared to the control. The band is still apparent in the next accumulation although the temperature of the sample has continued to change from −8 to −10°C. Also notice that this spectrum was accumulated between 15 and 30 seconds after the onset of the temperature step. Finally, the complete disappearence of the (36 Å)$^{-1}$ signal was attained in the fourth spectrum (Fig. 6A, "45" sec), that is after the LDL sample has remained more than 30 seconds at temperatures below the freezing point of the solution in which LDL is immersed. In order to determine whether the delay in the low temperature induced disappearance of the band paralleled the ice formation from the solution, we made simultaneous measurements of the low and the high angle regions of the scattering patterns, looking for the appearance of reflections due to the water crystallization. In this respect a weak signal started to appear in the "15" sec inserted spectrum of figure 6A. Since this intensity was the only observed in the (6 Å)$^{-1}$ to (2.5 Å)$^{-1}$ range of the reciprocal space it can be associated with the (3.75 Å)$^{-1}$ reflection of the cubic ice lattice.[15] Its intensity increased during the third acquisition period ("30" sec), finally attaining a constant maximum value in the "45" sec spectrum (by comparison with the spectra recorded subsequently but not shown in the figure). The reverse temperature sequence was also explored; the thermal step was applied from −10 to 2°C and as it is shown in figure 6B there was also a clear delay in the appearance of the (36 Å)$^{1-}$ scattering maximum which parallels the melting of the ice.

Much slower changes were observed during the crystallization process of cholesteryl linoleate. The transition was induced by rapidly cooling the lipid sample from 42 to −3°C, looking for the appearance of high angle reflections characteristic of the crystalline phase. Fig. 7 shows a view of several 10 sec counting spectra which were recorded sequentially one after the other alternating with no counting intervals of 40 sec between each spectrum. The first diffraction pattern (time "0" sec) was recorded at the end of the temperature step (T=3°C) and shows the (36 Å)$^{-1}$ sharp reflection characteristic of the smectic phase. After remaining more than 4 minutes at −3°C a new reflection centered af $\mathcal{S}$=(18 Å)$^{-1}$ started to appear (time "250" sec). The subsequent spectra show how this signal continued to increase in intensity with the simultaneous decrease of the (36 Å)$^{-1}$ reflection. At higher angles other weak diffraction maxima were also observed which are not shown because the horizontal magnification of the picture. The reverse trans-

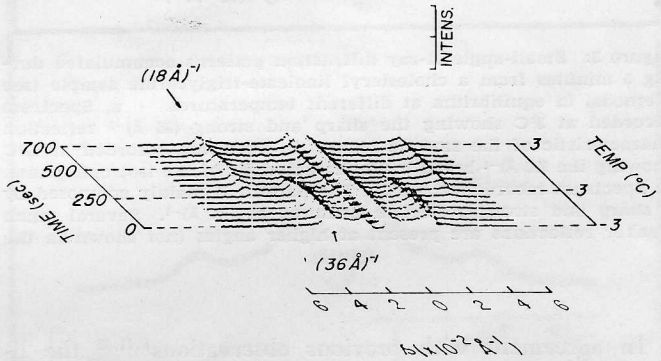

Figure 7: Overall view of a series of 15 experimental X-ray intensities from the neutral lipid mixture showing the kinetics of the smectic→crystalline isothermal transition. The spectra were sequentially recorded during 10 sec accumulation alternating with no counting intervals of 40 sec between the spectra. The first spectrum was recorded as a control. The (36 Å)$^{-1}$ and (18 Å)$^{-1}$ reflections characteristic of the smectic and crystalline phases are pointed by arrows. See legend of Fig. 5.

ition, namely the melting of the crystal was completed within a few seconds and it could not be followed. Fig. 8 shows the crystalline→isotropic phase transition. Since the smectic mesophase is monotropic[12,13,14] it did not appeared when the crystal was melted to the isotropic liquid state.

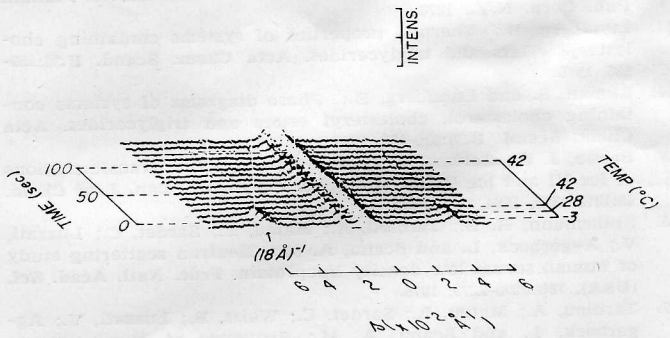

Figure 8: The kinetics of the crystalline→isotropic phase transition. The spectra were sequentially accumulated during 10 sec. The (18 Å)$^{-1}$ reflection characteristic of the crystalline phase is pointed by an arrow. Notice the absence of the (36 Å)$^{-1}$ reflection characteristic of the smectic phase. See legend of Fig. 5.

DISCUSSION

The relationship of the kinetics of transitions in mixtures of cholesteryl esters and triglycerides derived from LDL to the structure or dynamics of lipoproteins is important since in the body temperature range both the intact particles and the isolated lipids do undergo a similar thermal behaviour which has been associated to a smectic↔isotropic liquid transition exhibited by the cholesteryl esters.[6,7,8,9] In fact, Deckelbaum et al.[6,8] have proposed that the neutral lipids in LDL are located within the core of the particle arranged in three concentric layers similar to the smectic phase of pure cholesteryl esters. In their interpretation this would be the reason why the X-ray patterns of both systems show a scattering signal centered in the same angular region (36 Å)$^{-1}$.

Although the position sensitive detectors are capable of recording rapid structural changes, useful kinetic information can only be obtained if the time required for data collection is short as compared to the transition times. As it was shown here at body temperatures the detectors were not able to record the intermediate structures for LDL and neutral lipids. The transitions occurred respectively in less than 15 and 4 seconds which is too rapid to be followed with our detection method. These measurements might be improved by using more intense incident beams (for example, from storage rings) or samples which scatter more strongly. It is equally important to use temperature jumps which are faster than the structural changes. This condition was not either accomplished for the two systems studied here which were probably changing their structures significantly during the temperature jumps. For the low temperature transitions our experimental device was perfectly adequate since these processes were slower. Indeed, the isothermal crystallization of the pure neutral lipids occurred very slowly: a long delay of several minutes was observed before the appearance of the (18 Å)$^{-1}$ reflection characteristic of the crystalline phase. This signal continued to increase and at the end of the process its intensity was similar to that of the (36 Å)$^{-1}$ reflection previously observed in the smectic phase (see figures 4 and 7). This result suggests that a supercooling behaviour was probably followed by the development of a strongly hindered smectic-crystal boundary due to the highly viscous smectic phase. As it was shown in figure 6 the disappearance of the (36 Å)$^{-1}$ band from the X-ray patterns of LDL below 0°C is correlated with the ice formation.

The kinetics of the above processes were entirely different. As stated above the crystallization of the cholesteryl esters-triglycerides mixture was very slow, the (18 Å)$^{-1}$ reflection started to appear in about 5 minutes after the thermal step was applied. On the contrary in LDL samples the (36 Å)$^{-1}$ band disappeared in about 30 seconds simultaneously with the freezing of the solvent, in this case the (18 Å)$^{-1}$ reflection was not observed which —from the structural point of view— it is a significant difference between the two systems. An additional piece of information which also emphasizes this difference was provided by the experiments shown in figures 8 and 6: in the lipid system the reverse transition namely, from crystalline to isotropic never showed the (36 Å)$^{-1}$ reflection of the smectic phase since it is monotropic, however, in LDL the band centered in the same region of the reciprocal space appeared and disappeared going several times forward and backward across the transition. This different behaviour indicates that the arrangement of the cholesteryl esters within the core of the particle is not smectic.[6,8]

The results presented here confirm our previous observations[4,10] from which we have concluded that the disappearance at low temperatures of the (36 Å)$^{-1}$ band in LDL is not due to the crystallization of the cholesteryl esters but to a structural distortion produced at the surface of the lipoprotein particle by the freezing of the solvent. This conclussion supports the structural model recently proposed by V. Luzzati et al. (personal communication) which postulates that the neutral lipids are arranged in a cubic organization of spherical micellar elements which contain the steroid moiety of the cholesteryl esters embedded in a hydrocarbon matrix. This kind of arrangement is not surprising since under certain conditions the association of one dimensional lipid bilayers (containing phospholipids and cholesterol) with proteins induces the formation of three dimensional ordered lipoprotein structures (L. Mateu, work in progress).

Our present working hypothesis which is based in all the above observations is that the neutral lipids have to be arranged with a degree of symmetry higher than in the triple-layered model such as that proposed by Deckelbaum et al. The resolution of the LDL structure at very low temperatures (−10°C) will probably answer this point. (L. Mateu, work in progress.)

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REFERENCES

1. Gabriel A. and Dupont, Y.: A position-sensitive proportional detector for X-ray crystallography. **Rev. Sci. Instrum.** 43:1600-1602, 1972.

2. Dupont Y.; Gabriel, A.; Chabre, M.; Gulik-Krzywicki, T, and Schechter, E.: Use of a new detector for X-ray diffraction and kinetics of the ordering of the lipids in E. Coli membranes and model systems. **Nature (Lond), 238**:331-333, 1972.
3. Kirschner, D. A. and Caspar, D. L. D.: Myelin structure transformed by dimethylsulfoxide. **Proc. Natl. Acad. Sci. (USA), 72:** 3513-3517, 1975.
4. Mateu, L.; Kirchhausen, T. and Camejo, G.: Small-angle X-ray scattering and DSC studies on reversibly modified human serum low density lipoproteins. **Biochemistry, 17 (8)**:1436-1440, 1978.
5. Mateu, L.; Tardieu, A.; Luzzati, V.; Aggerbeck, I. and Scanu, A. M.: On the structure of human serum low density lipoproteins. **J. Molec. Biol., 70**:105-116, 1972.
6. Deckelbaum, R. J.; Shipley, G. G.; Small, D. M.; Lees, R. S. and George, P. K.: Thermal transitions in human plasma low density lipoproteins. **Science (Wash. D. C.), 190**:392-394, 1975.
7. Mateu, L.; Kirchhausen, T.; Padrón, R. and Camejo, G.: Small-angle X-ray scatttering study of human serum low density lipoproteins with differential reactivity for an arterial proteoglycan. **J. Supramolec. Structure 7**:435-442, 1978.
8. Deckelbaum, R. J.; Shipley, G. G. and Small, D. M.: Structure and interactions of lipids in human plasma low density lipoproteins. **J. Biol. Chem., 252**:744-754, 1977.
9. Muller, K.; Laggner, P.; Glatter, O. and Kostner, G.: The structure of human plasma low density lipoprotein B. **Eur. J. Biochem., 82**:73-90, 1978.
10. Mateu, L.; Kirchhausen, T. and Camejo, G.: A low temperature structural transition in human serum low density lipoproteins. **Biochim. Biophys. Acta 487**:243-245, 1977.
11. Kirchhausen, T.: Estudio con Rayos-X de la estructura de algunas lipoproteínas y la enfermedad de Tay-Sachs. **Ph. Sc. Tesis C.E.A.,** I.V.I.C., 1977.
12. Small, D. M.: The physical state of lipids of biological importance: cholesteryl esters, cholesterol, triglycerides. In surface Chemistry of Biologycal Systems. **M. Blank Editor. Plenum Pub. Corp.** N.Y., 1970.
13. Lundberg, B.: Thermal properties of systems containing cholesteryl esters and triglycerides. **Acta Chem. Scand. B.30**:150-156, 1976.
14. Ekman, S. and Lundberg, B.: Phase diagrams of systems containing cholesterol, cholesteryl esters and triglycerides. **Acta Chem. Scand. B.30**:825-830, 1976.
15. Bertie, J. E.; Calvert, L. D. and Whalley, E.: Transformations of Ice VI and Ice VII at atmospheric pressure. **Can. J. of Chem. 42**:1373-1378, 1964.
16. Stuhrmann, H. B.; Tardieu, A.; Mateu, L.; Sardet, C.; Luzzati, V.; Aggerbeck, L. and Scanu, A. M.: Neutron scattering study of human serum low density lipoprotein. **Proc. Natl. Acad. Sci. (USA),.72b**:2270-2273, 1975.
17. Tardieu, A.; Mateu, L.; Sardet, C.; Weiss, B.; Luzzati, V.; Aggerbeck, L. and Scanu, A. M.: Structure of human serum lipoproteins in solution. II. Small-angle X-ray scattering study of HDL_3 and LDL. **J. Molec. Biol., 101**:129-153, 1976.