

The Storage Lipids in Tangier Disease

A PHYSICAL CHEMICAL STUDY

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ABSTRACT The physical states and phase behavior of the lipids of the spleen, liver, and splenic artery from a 38-yr-old man with Tangier disease were studied. Many intracellular lipid droplets in the smectic liquid crystalline state were identified by polarizing microscopy in macrophages in both the spleen and liver, but not in the splenic artery. The droplets within individual cells melted sharply over a narrow temperature range, indicating a uniform lipid composition of the droplets of each cell. However different cells melted over a wide range, 20–53°C indicating heterogeneity of lipid droplet composition between cells. Furthermore, most of the cells (81%) had droplets in the liquid crystalline state at 37°C. X-ray diffraction studies of splenic tissue at 37°C revealed a diffraction pattern typical of cholesterol esters in the smectic liquid crystalline state. Differential scanning calorimetry of spleen showed a broad reversible transition from 29–52°C, with a maximum mean transition temperature at 42°C, correlating closely with the polarizing microscopy observations. The enthalpy of the transition, 0.86 ± 0.07 cal/g of cholesterol ester, was quantitatively similar to that of the liquid crystalline to liquid transition of pure cholesterol esters indicating that nearly all of the cholesterol esters in the tissue were free to undergo the smectic-isotropic phase transition.

Lipid compositions of spleen and liver were de-

termined, and when plotted on the cholesterol-phospholipid-cholesterol ester phase diagram, fell within the two phase zone. The two phases, cholesterol ester droplets and phospholipid bilayers were isolated by ultracentrifugation of tissue homogenates. Lipid compositions of the separated phases approximated those predicted by the phase diagram. Extracted lipids from the spleen, when dispersed in water and ultracentrifuged, underwent phase separation in a similar way. Thus (a) most of the storage lipids in the liver and spleen of this patient were in the liquid crystalline state at body temperature, (b) the phase behavior of the storage lipids conformed to that predicted by lipid model systems indicating lipid-lipid interactions predominate in affected cells, (c) lipid droplets within individual cells have similar compositions, whereas droplet composition varies from cell to cell, and (d) cholesterol ester does not accumulate in the splenic artery. Since Tangier patients lack high density lipoprotein, we conclude that high density lipoprotein-mediated cholesterol removal from cells is essential only for those cells which have an obligate intake of cholesterol (macrophages).

INTRODUCTION

Tangier disease is a rare hereditary disorder characterized by severe deficiency of high density lipoproteins and especially their major apoprotein, apo A-I (1). Although affected patients have an abnormally low serum cholesterol, there is widespread tissue accumulation of cholesterol ester in the spleen, liver, tonsils, bone marrow, intestine, thymus, and peripheral nerves (2, 3). A particular cell type, the tissue macrophage is the site of most of the lipid deposition. Unlike atherosclerotic lesions which also contain large amounts of intracellular cholesterol ester, there is no accompanying fibrosis or gross distortion of tissue architecture. The pathogenesis of lipid accumulation in

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Tangier disease, and its relationship to the plasma lipoprotein abnormalities have not been elucidated.

The physical state of the storage lipids may be an important factor in their accretion. For example, cholesterol esters in the liquid state may be more readily hydrolyzed and removed from tissues, whereas liquid crystalline or crystalline esters may not be as readily mobilized. However, in Tangier disease, the *in vivo* physical state of the lipid is not known, as neither tissues nor their isolated lipids have been studied by physical techniques at body temperature. Ferrans and Frederickson (3) have suggested that a portion of the cholesterol esters are crystalline, although they recognized that artifactual production of crystals during tissue processing might have occurred. Previous analyses of the lipids from tonsil, lymph node, and spleen of patients with Tangier disease have demonstrated the presence of three major classes of lipid: cholesterol ester, phospholipids, and free cholesterol (4-6). If the compositions are considered in terms of the model phase diagram of these lipids (7), it may be predicted that two distinct lipid phases are present in affected organs, a cholesterol ester droplet phase, and a phospholipid liquid crystalline phase. We have investigated the phase behavior of the lipids in Tangier disease, looking at the lipids by physical techniques *in situ* in the tissues, and by isolating and characterizing the lipid phases. Further, since the physical state of lipids may affect their metabolism and so favor their intracellular accumulation, we have studied the physical states of the lipids of the spleen, liver, and splenic artery in a patient with Tangier disease. Lipid compositions of whole tissue and the isolated storage lipids were determined and results analyzed in terms of the phase diagram of model lipid systems. As well, the physical states of lipids within individual cells were studied as a function of temperature, and evidence was obtained of chemical homogeneity of lipid droplets within each cell but heterogeneity of lipid droplet composition from cell to cell.

METHODS

Patient and tissue material. The patient, a 38-yr-old male, is the 18th reported case of Tangier disease, and was the subject of a recent case report (8). His serum cholesterol and triglyceride averaged 42 and 297 mg/dl, respectively, and high density lipoprotein cholesterol was 0. Because of increasing abdominal discomfort and evidence of hypersplenism, a splenectomy was performed as well as an intra-operative wedge biopsy of the liver. Fully informed consent to study tissues had been obtained before surgery. Immediately after excision, a small section of spleen was removed for electron microscopy and histology. To wash the blood out of the spleen, it was perfused at a rate of 30 ml per minute with 500 ml of modified Hank's solution (9) (NaCl, 6.22 g; KCl, 0.4 g; $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.06 g; KH_2PO_4 , 0.06; NaHCO_3 , 0.35 g; glucose, 0.9 g; distilled

water to 1,000 ml; pH 7.2) followed by 600 ml of modified Hank's solution containing 0.8% collagenase, 1.0% hyaluronidase (Worthington Biochemical Corp., Freehold, N. J.) and 1% human serum albumin. The enzymes were used to facilitate the isolation of individual foam cells as described below. Portions of spleen were then taken for polarizing microscopy, x-ray diffraction, differential scanning calorimetry, chemical analyses, dry weight and lipid content determination, and for lipid phase separation studies. Similar studies were performed on liver, splenic artery, and normal control spleens.

Cell melting. Small amounts of spleen or liver tissue were gently minced on a glass slide, excess tissue was removed, and a coverslip was placed over the cells adhering to the glass. The boundaries of the droplet-laden cells were easily distinguished both by ordinary light microscopy and by Nomarski microscopy (Figs. 1a and 2). A Zeiss standard WL microscope (Carl Zeiss, Inc., New York) equipped with a heating and cooling stage was used to assess the physical states of the lipids and to determine their melting points (10). The heating rate was $2^\circ\text{C}/\text{min}$, and melting temperatures were determined to $\pm 0.5^\circ\text{C}$.

Differential scanning calorimetry (DSC).¹ A Perkin-Elmer DSC-II differential scanning calorimeter (Perkin-Elmer, Norwalk, Conn.) was used to study phase transitions in fresh tissue or isolated lipid droplets. 50-80 mg of spleen, or its isolated lipid droplets, were hermetically sealed in stainless steel Perkin-Elmer volatile pans. Reference pans were loaded with appropriate amounts of water to balance the water content of the sample. Heating and cooling rates were $10^\circ/\text{min}$. Initial scans were performed between 0 and 55°C with subsequent cooling to -73°C before reheating. The enthalpy of transitions, ΔH , was calculated by relating area under the peak to that of a known indium standard.

X-ray diffraction. An Elliott toroidal camera and nickel-filtered $\text{CuK}\alpha$ radiation from an Elliot GX6 rotating anode generator (Baird & Tatlock, London, England) were used for all x-ray diffraction experiments. Samples were positioned between Mylar windows in a Teflon "o" ring within a controlled temperature sample holder.

Dry weight and lipid content. Portions of Tangier and control spleen were weighed on a Mettler H2O balance (Mettler Instrument Corp., Princeton, N. J.) before and after vacuum desiccation for 48 h over P_2O_5 at room temperature. Lipid content was determined after quantitative recovery of the chloroform phase of a Folch extraction, followed by evaporation of the chloroform to a constant dry weight.

Chemical analysis. Tissues were homogenized in 20 vol of chloroform-methanol 2:1 (vol:vol) and the extracted lipids were washed with 0.04% CaCl_2 according to Folch et al. (11). Lipid classes were separated and measured by quantitative thin-layer chromatography (TLC) as previously described (12, 13). Cholesterol esters were isolated by preparative TLC with a solvent system of hexane-diethyl ether 94:6 (vol:vol). Lipid bands were visualized with ultraviolet light after spraying with 2,7-dichlorofluorescein (Applied Science Labs, Inc., State College, Pa.). The cholesterol ester bands were scraped and the lipid eluted with chloroform through a scintered glass filter. Fatty acids were saponified in ethanol-KOH at 80°C (14), and methylation accomplished with 14% BF_3 in methanol at 80°C (15). Methyl esters were quantitated with a Packard model 804 gas-liquid chromatograph (Packard Instru-

¹ Abbreviations used in this paper: DSC, differential scanning calorimetry; TLC, thin-layer chromatography; Tm, maximum mean transition temperature.

ments Co., Inc., Downers Grove, Ill.) equipped with a hydrogen flame ionization detector. A 6-foot column packed with 10% SP2300 on 100/120 Supelcoport (Supelco Inc., Bellefonte, Pa.) was operated at 215°C. Relative amounts of methyl esters were calculated by multiplying peak heights by retention times.

Separation and characterization of lipid phases of spleen and liver. Tissues were homogenized for 2 min in distilled water by using a Kontes Duall glass-glass tissue grinder (Kontes Co., Vineland, N. J.). Homogenates were centrifuged at 24°C for 1 h at 100,000 g. The floating layer (the lipid droplet phase) of the spleen homogenate was recovered with a

spatula, and portions taken for polarizing microscopy, DSC, x-ray diffraction, quantitative TLC, and gas-liquid chromatography of the cholesterol esters. To compare the composition and microscopic appearance of droplets isolated by a less disruptive technique, a small quantity of partly enzymatically digested splenic tissue was dispersed in water by aspirating repeatedly into a Pasteur pipette and the suspension was centrifuged at 200 g for 45 min at 23°C allowing intact lipid droplets to form a floating layer. The liver homogenate floating layer was recovered with a Pasteur pipette, and similar studies were carried out. Pellets were resuspended in water, recentrifuged, then examined by

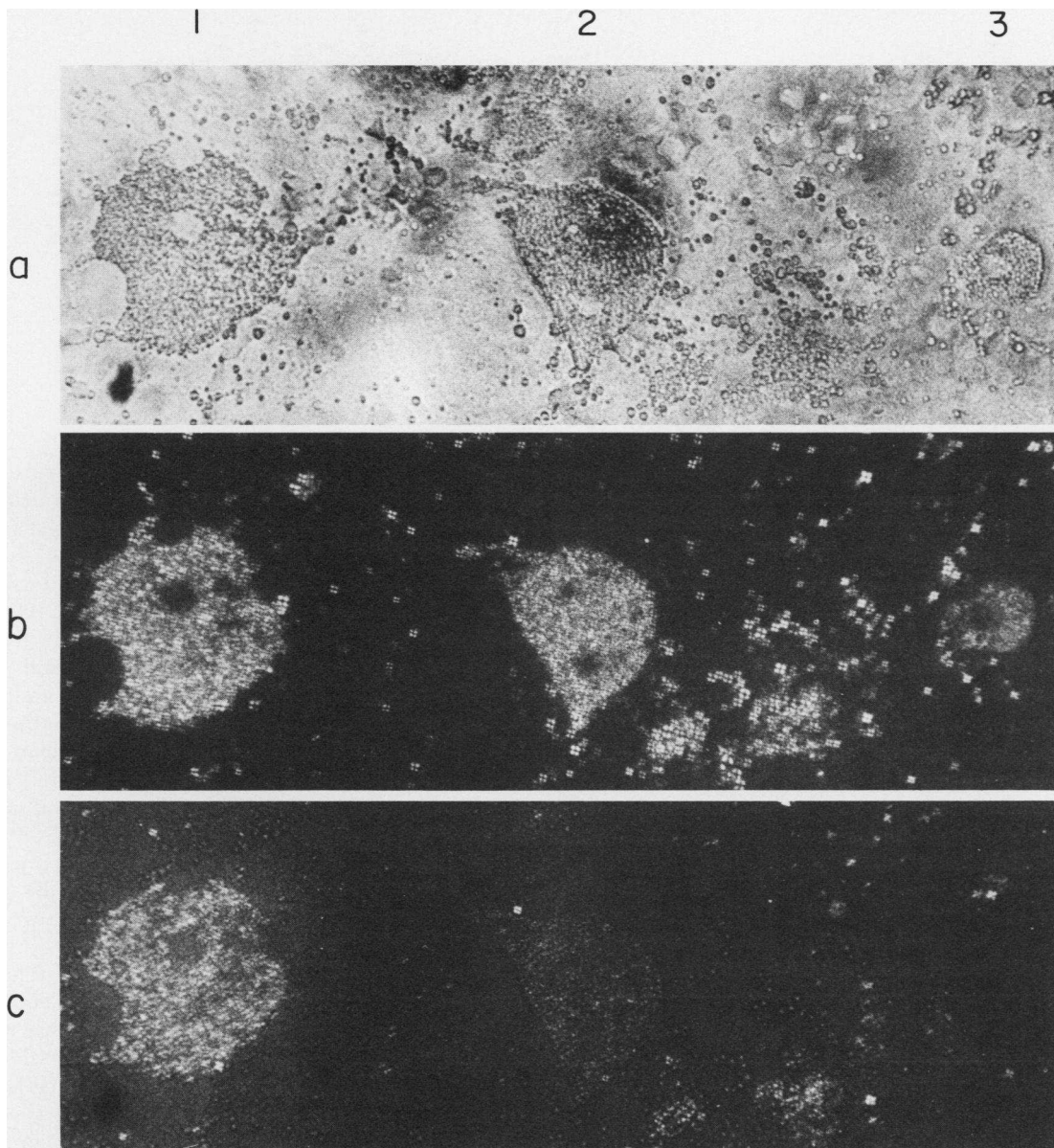


FIGURE 1 Spleen foam cells in Tangier disease. Spaces in cell represent nuclei. Same field, magnification $\times 400$ (a) ordinary light, 20°C, (b) crossed polarizers 20°C, and (c) crossed polarizers 48°C. At 48°C, droplets in cell 1 are still birefringent, those in cell 2 are in the process of melting, and those in cell 3 have finished melting and are isotropic liquids.

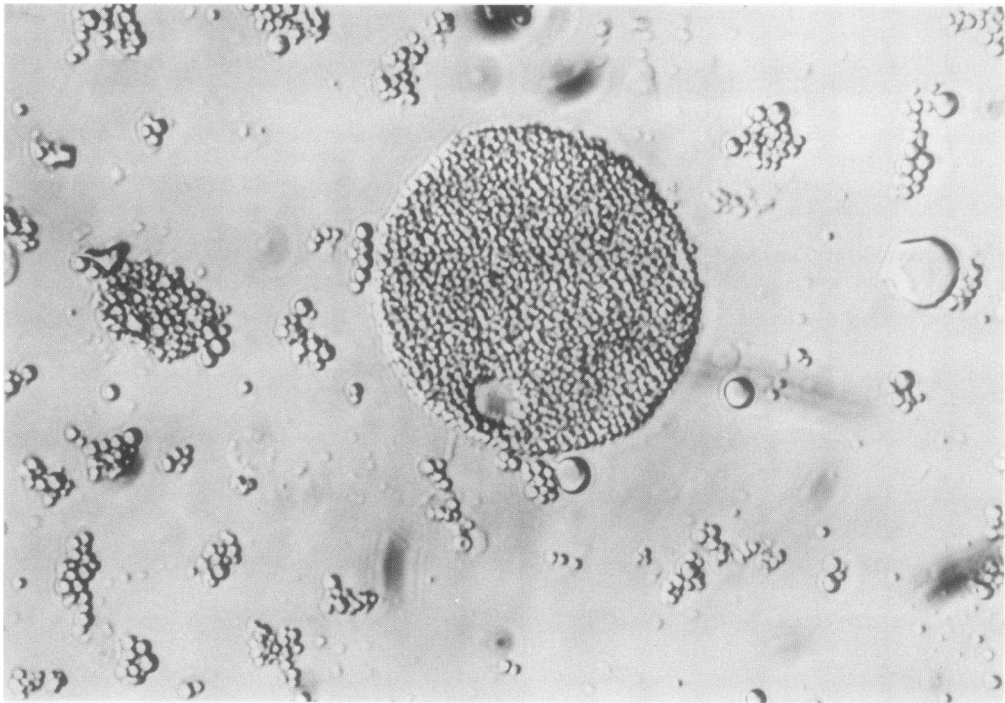


FIGURE 2 Spleen foam cell 20°C, magnification $\times 960$, Nomarski optics.

polarizing microscopy, and the lipids analyzed by quantitative TLC.

Phase behavior of extracted lipids. 20 mg of Tangier and control spleen lipids in chloroform was placed in a glass tube with a central constriction and the solvent evaporated under a stream of dry N_2 . Removal of organic solvents was completed by drying *in vacuo* overnight. 100 μ l of distilled water was added, the tube flushed with N_2 , and sealed. Lipids were dispersed into the water by centrifuging back and forth through the constriction at 55°C. The equilibrated mixture was drawn into a 70- μ l capillary tube which was then sealed and centrifuged at 40,000 g at 39°C for 17 h. The separated layers were recovered, and examined with polarizing microscopy, and the lipid composition of each layer was determined.

RESULTS

At operation the spleen was enlarged to three times normal size and the surface had a red and yellow speckled appearance. Yellow fatty streaking was visible through the serosal surface of the small bowel. The liver appeared normal.

Cellular lipid droplet melting. 81 individual spleen foam cells were studied by polarizing microscopy. Cells were 7–15 μ m in diameter and were packed with liquid crystalline droplets (Fig. 1). One or two spaces devoid of droplets represented the nuclei of cells as shown by Nomarski optics (Fig. 2). Grossly irregular borders of some of the cells were due to spreading on contact with glass, a characteristic of macrophages (16). Each cell was studied degree by

degree from 15 to 60°C. All cell droplets were liquid crystalline at 18°C and a positive sign of birefringence indicated the smectic state (10, 17). No crystals were observed in any of the fresh tissues. On heating, droplets within individual cells melted sharply, usually over a 1–2°C temperature range. However droplets of different cells melted at widely different temperatures, from 20 to 53°C (Figs. 1 and 3). Although a rare cell had droplets which melted by 20°C, greater than 95% of cells melted over the range 30–53°C (Fig. 3, upper portion). Moreover, most droplets had melting points higher than body temperature, since at 37°C only 19% of the cells had droplets in the liquid state (Fig. 3, lower portion). At 40°C, there was a maximum mean transition temperature (T_m) in the number of cells with droplets in the process of melting and 41°C was the temperature at which half the cells had melted droplets.

After melting, the droplets within cells tended to coalesce, unlike their behavior in the liquid crystalline state where they remained discrete. The phase change from liquid crystalline to liquid was reversible, and the sequence of return of birefringence of cells could be predicted from their order of melting.

DSC. DSC of splenic tissue revealed a broad reversible transition between 29 and 52°C, with a maximum T_m at 42°C (Fig. 4). This correlated well with the melting range (Fig. 3, upper portion) and T_m for cell melting observed by polarizing microscopy. The

enthalpy (ΔH) of the transition, 0.86 ± 0.07 cal/g cholesterol ester ($n = 6$ heating or cooling runs) correlated closely with the enthalpy of the liquid crystalline to liquid transition of pure cholesterol esters (18). This strongly suggests that most of the cholesterol ester in the tissue is free to undergo the smectic to isotropic transition. Further, only 17% of the transition had taken place by 37°C , which correlated with the microscopic observation that 19% of the cells had melted lipid droplets at 37°C . Cooling the tissue to -73°C resulted in a much larger transition between 26 and 55°C , with a $\Delta H = 7.1$ cal/g (crystal-liquid transition of pure cholesterol esters $\Delta H = 8-11$ cal/g [18]). Thus, most of the cholesterol ester of the tissue could crystallize if cooled to -73°C .

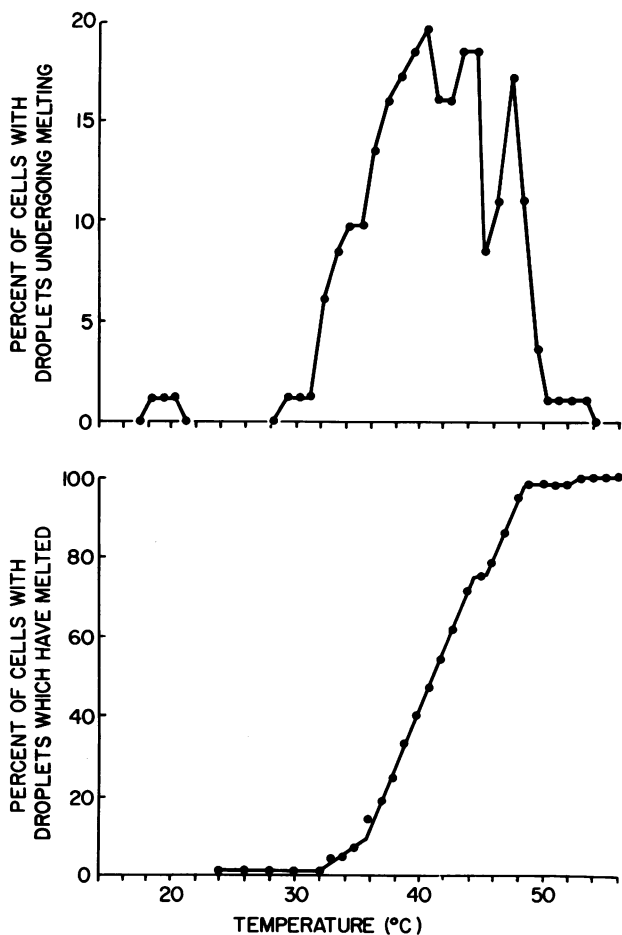


FIGURE 3 Above, distribution of cells with lipid droplets in the process of melting. 81 individual cells were studied by hot stage polarizing microscopy. Melting range is $20-53^\circ\text{C}$. At 40°C , the maximum number of cells have droplets in the process of melting. Below, cumulative plot of number of cells with lipid droplets in the liquid state, as a function of temperature. At 37°C , only 19% of the cells have droplets which have melted. 50% of the cells have melted droplets at 41°C .

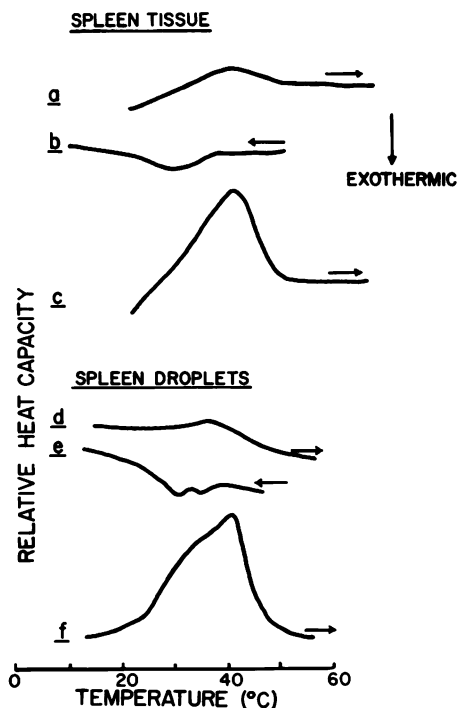


FIGURE 4 Differential scanning calorimeter curves of heating (\rightarrow) and cooling (\leftarrow) runs of splenic tissue and isolated splenic lipid droplets. 50–80 mg of tissue was sealed in a stainless steel pan and heated or cooled at a rate of $10^\circ\text{C}/\text{min}$. If no transition occurs then the base line will be flat, but if a transition occurs, such as melting of the lipid, then heat will be absorbed and the curve will deviate above the base line, as in curves a, c, d, and f. Deviations above the base line are endothermic. If the lipid freezes on cooling, as in b and e, then the process is exothermic. The peak of the endothermic or exothermic deviation from base line gives an estimate of the midpoint of the melting or freezing process and the area under the curve gives the enthalpy (ΔH) of the transition. a, b, d, and e are low enthalpy, reversible liquid crystalline-liquid transitions. c and f are crystal to liquid transitions produced after cooling sample to -73°C .

Droplets separated from homogenized spleen by ultracentrifugal floatation also demonstrated a reversible transition between 24 and 49°C . T_m of the transition was 39°C , and ΔH was 0.92 ± 0.07 cal/g ($n = 4$ heating or cooling runs). Cooling to -73°C resulted in crystallization of the cholesterol esters (ΔH of fusion 9.09 cal/g). The separated cholesterol ester phase exhibited both a cholesteric (10) and a smectic transition on cooling at 35 and 30°C , respectively. The cholesteric phase was not observed in the *in situ* droplets.

X-ray diffraction. The x-ray diffraction pattern obtained from spleen at 20°C confirmed the smectic liquid crystalline state of the cholesterol ester droplets. The sharp narrow angle line at 35.3\AA and diffuse band centered at 5\AA was a pattern

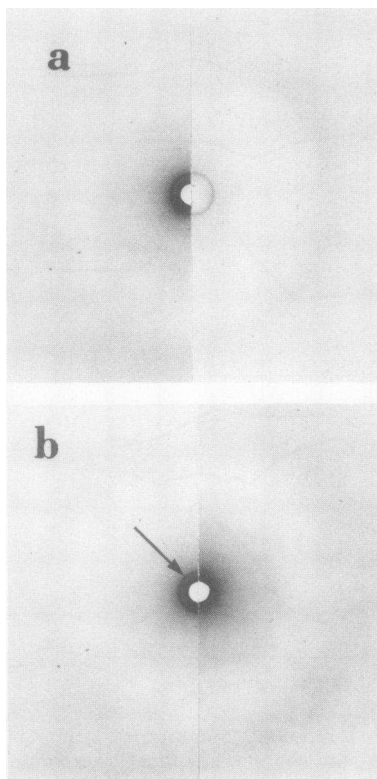


FIGURE 5 (a) X-ray diffraction pattern of spleen at 20°C (left) and cholesteryl oleate/cholesteryl linoleate 6:4 (mol:mol) in the smectic liquid crystalline state (right). (b) X-ray diffraction pattern of spleen at 20°C (left) and at 50°C (right). The 35.3Å spacing (arrow) has disappeared at 50°C.

similar to that produced by a mixture of pure cholesteryl oleate/cholesteryl linoleate 6:4 (mol/mol) in the smectic state (Fig. 5a). At 37°C, the 35.3Å line was weaker, but still present; at 50°C the narrow angle line was replaced by a diffuse band of weak intensity (Fig. 5b). The total lipids extracted from the spleen also showed a smectic pattern at 37°C. X-ray diffraction studies of liver tissue also showed a smectic cholesterol ester pattern.

Phase separation

(a) *Homogenates of spleen and liver.* The lipid compositions of the spleen and liver are shown in Table I. Lipids formed 21.6% of the dry weight of the spleen (control = 10.8%). The compositions, plotted on the phase diagram in Fig. 6a, fall within the 2 phase zone, as we would expect from our identification in these tissues of the cholesterol ester droplet phase and the phospholipid membrane phase. To separate the lipid phases, spleen or liver were homogenized in distilled water and centrifuged for 6×10^6 g-min. The spleen homogenate separated into a thick solid orange-colored floating layer which could be removed as an intact disk with a spatula. The pellet consisted of a whitish layer overlying a dark red layer. The liver floating layer was smaller, and light yellow in color.

Compositions of the layers (Table II) show that almost complete lipid phase separation was achieved in spite of the presence of nonlipid components in the homogenates. Lipid droplets separated from the spleen by less harsh methods, were similar in size and melting behavior to those within intact cells and had the same composition as the spleen homogenate floating layer. Plotted on the phase diagram (Fig. 6b) the compositions of separated layers were close to the respective one phase zones predicted by the phase diagram.

(b) *Total lipid extract of spleen.* To demonstrate similar phase separation in a pure lipid system, the extracted spleen lipids were dispersed in distilled water, and phase separation achieved by ultracentrifugation (Fig. 7). Three lipid layers were separated: (1) a clear orange colored oil, (2) a yellow emulsion layer, and (3) a whitish opaque bottom layer below the clear intervening water layer. The lipid composition of each layer (Fig. 7) shows that layer 1 is the cholesterol ester oil phase, layer 3 is the phospholipid bilayer phase, and layer 2 contains both phases. Since the separated phases are derived from the total lipids of the spleen homogenate, they must

TABLE I
Lipid Composition (Weight Percent) of Tissues in Tangier Disease and Control Spleen

	Cholesterol	Triglyceride	Cholesterol ester	Sphingomyelin	Lecithin	Phosphatidyl-ethanolamine
Tangier spleen	9.4	1.4	67.2	4.0	12.1	6.0
Control spleen* $n = 3$	25.0±2.0	6.4±1.2	4.7±0.6	9.3±1.1	29.3±1.7	22.2±0.3
Tangier liver	5.3	24.9	24.2	6.8	24.8	14.1
Tangier splenic artery	24.0	12.2	6.0	28.6	29.2	—

* ±SEM.

lie on a straight line joining the homogenate composition. The relative amounts of the cholesterol ester and phospholipid phases in the spleen are inversely proportional to their respective distances from the homogenate along the line. Thus, the cholesterol ester phase accounts for 70.1% of the total lipids, and the phospholipid phase 29.9%.

Control spleen. Three control spleens examined by polarizing microscopy, had rare lipid droplets which were isotropic even when cooled to 0°C. No DSC transitions were present between 0 and 50°C. The mean lipid composition (Table I) plotted on the phase diagram (Fig. 6a) shows that the phospholipid membrane phase accounted for almost all of the lipid. Phase separation experiments as described above yielded a minute floating layer which was composed mainly of triglyceride. The content of cholesterol ester in the control spleens was less than 1/60th of the Tangier spleen.

Splenic artery. The intimal surface of the splenic artery was lesion-free when examined with a dissecting microscope (magnification $\times 40$). No evidence of intimal lipid accumulation was found by polarizing microscopy. The lipid composition (Table I) when plotted on the phase diagram (Fig. 6a) shows that very little of the cholesterol ester phase was present.

Gas-liquid chromatography. The relative composition of the individual cholesterol esters of the spleen and liver, and of the isolated spleen lipid droplet phase are shown in Table III. Cholesteryl oleate is the predominant ester, followed by palmitate, stearate, linoleate, and arachadonate. The saturated and monounsaturated esters comprised 78.6% of the total cholesterol esters of the spleen.

DISCUSSION

With three separate physical techniques, X-ray diffraction, DSC, and polarizing light microscopy we have shown that most of the storage lipid in a patient with Tangier disease was in the smectic liquid crystalline state at 37°C. X-ray diffraction studies of spleen and liver tissue showed a diffraction pattern typical of smectic liquid crystals of mono and di-unsaturated C_{18} cholesterol esters, and mixtures of esters such as those found in human fatty streaks (13) and serum low density lipoproteins (19). Both DSC and hot stage polarizing microscopy revealed that at 37°C a large proportion of the cholesterol ester droplets (about 82%) were in the liquid crystalline state. Since the lipid compositions in previously reported cases of Tangier disease (4–6) were very similar to our own, it is likely that much of the stored cholesterol ester in those cases was also liquid crystalline at body temperature.

The coalescence of droplets after melting is further evidence of their existence in the liquid crystalline

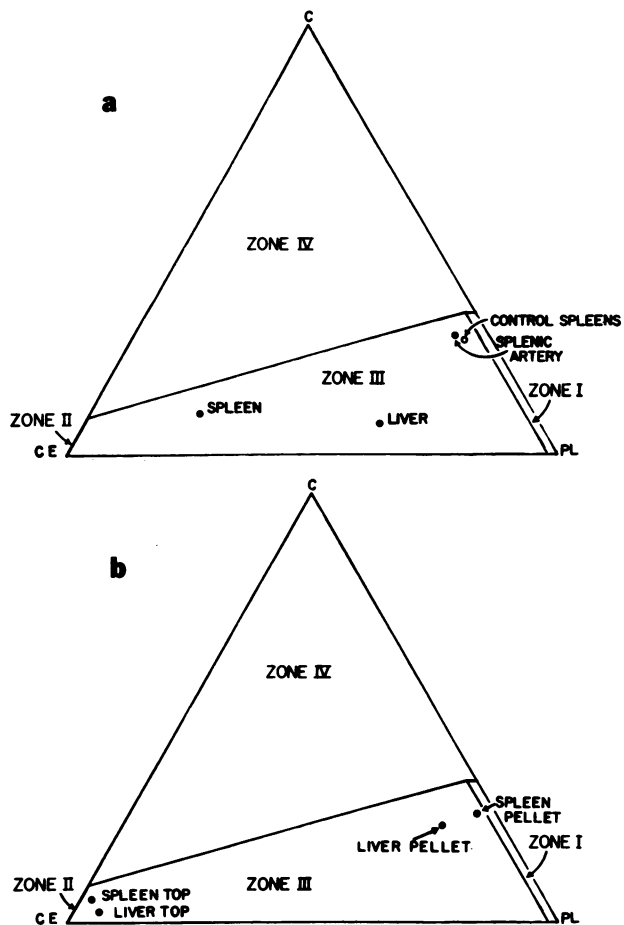


FIGURE 6 (a) Lipid compositions of Tangier spleen, liver, splenic artery, and control spleens, plotted on the phase diagram of cholesterol (C), phospholipid (PL), and cholesterol ester (CE). (7). Zone I is a one phase zone of phospholipid liquid crystals in which up to 33% (wt/wt) cholesterol and 2% cholesterol ester can be solubilized. The essential structural feature of this phase is that of bimolecular leaflets of phospholipid separated by layers of water. The cholesterol is interdigitated between the phospholipid molecules (7). Zone II is also a one phase zone of cholesterol ester droplets in which 3–8% cholesterol, but almost no phospholipid can be solubilized. The phase can be liquid or liquid crystalline depending on the temperature and the fatty acyl chain composition of the esters (10). In general the more unsaturated the fatty acyl chain the more likely it will be a liquid at body temperature. Zone III is a two phase zone in which both phospholipid liquid crystals and cholesterol ester droplets coexist and Zone IV contains, in addition, a third phase, cholesterol monohydrate crystals. See reference 7 for derivation of phase diagram. (b) Lipid compositions of separated phases of spleen and liver.

state *in vivo*. The nonmembrane-bound droplets do not coalesce on contact before melting, and therefore have a surface stabilizing monolayer, probably composed of phospholipid. The amount of phospholipid, acquired *in vivo*, is sufficient to cover their surface. However, when the cholesterol ester melts,

TABLE II
Lipid Composition (Weight Percent) of Tissue Homogenate Fractions

	Cholesterol	Triglyceride	Cholesterol ester	Sphingomyelin	Lecithin	Phosphatidyl-ethanolamine
Spleen top	5.0	1.0	92.0	0.4	1.5	—
Spleen pellet	25.5	—	6.5	9.6	41.0	17.4
Liver top	2.1	24.9	69.5	0.5	3.0	—
Liver pellet	20.4	—	20.9	4.8	12.6	42.0

there is an abrupt increase in its volume (20). The available phospholipid becomes insufficient to form an intact monolayer around the droplets, and when areas of exposed cholesterol ester come into contact, the droplets fuse.

The composition of the isolated lipid droplets offers an explanation for the melting points of the droplets. Like fatty streak droplets, they are composed mostly of cholesterol esters, with small amounts of solubilized free cholesterol and triglyceride (21, 22). The melting temperature of cholesterol esters with a constant fatty

acid chain length falls as the number of cis double bonds increases (10). For binary mixtures of cholesterol esters, the mesophase transition temperatures are linear functions of their relative compositions (23).² Knowing the relative content of the individual cholesterol esters of the Tangier droplets (Table III) one can calculate from the smectic transition of individual esters (10) what the smectic transition of the cholesterol esters present in the droplet should be. A transition temperature of 46°C is calculated for the mixture of droplet esters. However, the droplets also contain triglyceride and free cholesterol and these components affect cholesterol ester melting (10). The 1% triglyceride content of the droplets lowers the smectic melting temperature by about 1°C (24), and the 5% free cholesterol lowers it a further 4°C.² Therefore, the average predicted melting temperature of the Tangier lipid droplets is 41°C, correlating very closely with that found by DSC (42°C) and polarizing microscopy (41°C).

The problem of cholesterol ester accumulation in cells, such as those of Tangier disease or atherosclerotic fatty streaks has not been solved metabolically, biochemically, or physically. We suggest that the physical states of lipids should be considered in any future theories concerning the pathogenesis of these diseases. In Tangier cells a major fraction of the deposited cholesterol ester was in a liquid crystalline or highly ordered state. Further, and in line with the observations of Hillman and Engelman on clusters of lipid deposits in fatty streaks seen after freezing and sectioning (25), all of the lipid droplets within an individual cell undergo melting over a narrow temperature range. Since the melting temperature is very sensitive to lipid composition, the droplets within a given cell must have very similar lipid compositions. While this is not entirely surprising, it does mean that one cannot have two different kinds of lipid accumulations within the cell. For instance, a lysosomal deposit of partially catabolized low density lipoprotein high in cholesterol linoleate (low melting) does not coexist in the same cell with storage

² Small, D. M. Unpublished observations.

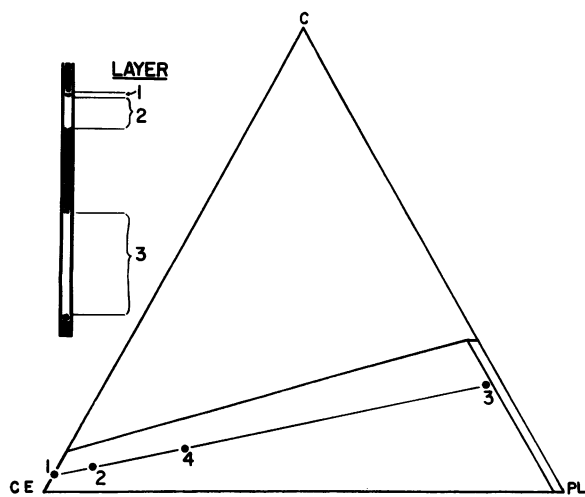


FIGURE 7 Phase behavior of extracted Tangier spleen lipids. Lipids extracted from Tangier spleen by the method of Folch et al. (11) were dried, suspended in water, equilibrated, drawn into a capillary tube, and centrifuged 17 h at 40,000 g at 39°C. The appearance of the separated phases is shown in capillary tube at left. Point 4 is the original composition of the extracted lipids plotted on triangular coordinates as in Fig. 6 and points 1–3 define the compositions of layers in the capillary tube. Layer 1 is a single phase of orange-colored oil rich in cholesterol ester. (CE) Its composition (point 1) defines the composition of the cholesterol ester phase. Layer 2 is an emulsion of incompletely separated cholesterol ester and phospholipid (PL). Between layer 2 and 3 is a water layer devoid of lipid. Layer 3 is a cloudy suspension of the phospholipid bilayer phase. Point 3 defines the composition of the phospholipid phase.

deposits high in cholesterol oleate (high melting). Two such droplets would have melting points differing by several degrees and this was not seen in any cell. Furthermore, the droplets within a given cell cannot contain large differences in the quantities of impurities (such as triglyceride or free cholesterol) or the droplets would vary appreciably in melting temperatures. Therefore, the observation strongly supports the idea that all of the droplets within a cell have a similar composition and therefore probably arise through the same metabolic mechanism.

However, the marked differences in the melting point from cell to cell in a given microscopic field, both in a Tangier spleen impression preparation and in foam cells of fatty streaks (26) suggest that the lipids have been produced by different cell types, by a different mechanism, or at a different time. This fascinating observation from the point of view of cellular biology, will need to be taken into account in the pathogenesis of any disease in which lipids accumulate.

This report is the first in which the physical state, in contrast to the chemical composition, of the storage lipids in Tangier disease has been investigated at body temperature in tissue which has not been subjected to procedures which drastically alter the physical state (cooling, freezing, fixing, imbedding, staining, etc.). Most of the stored cholesterol esters are in the smectic liquid crystalline state. There are many properties of the liquid crystalline state which are strikingly different from the liquid state. For instance, the smectic liquid crystalline state is an ordered state; the long axes of the esters are all arranged in the same direction. Furthermore, it is a layered state in which the cholesterol ester molecules are extended and the steroid moieties of the cholesterol ester are lined in distinct rows with the hydrocarbon chains in adjacent rows. This ordered state might make it quite difficult for an enzyme to orient itself to the ester bond for hydrolysis. Second, the viscosity of the liquid crystal droplet is considerably different from the viscosity of a true liquid (27) and, as a result, the diffusion of molecules through the liquid is much faster than diffusion in the liquid crystal. Furthermore, in the liquid crystal the diffusion rates are anisotropic, being greater in one direction than in another. Thus, if one thinks from a physical-biochemical point of view, the arrangement of the substrate may be very important in the rate of an enzymatic reaction such as ester hydrolysis. Since cholesterol is removed from macrophages only in the free form (28), impaired hydrolysis of cholesterol ester would decrease its rate of efflux from the cell. We have found that some human fatty streak lesions also have a high proportion of cholesterol ester droplets in the smectic liquid crystalline state at 37°C (26); thus similar physical-chemical factors

TABLE III
Cholesterol Ester Fatty Acids of Tangier Liver and Spleen (Weight Percent)

	Spleen	Spleen top layer	Liver
C14:0	0.7	0.5	—
C16:0	15.7	12.8	6.5
C16:1	2.2	1.2	0.5
C18:0	7.1	5.7	8.7
C18:1	49.8	51.0	65.4
C18:2	12.0	14.4	11.6
C18:3	0.3	0.3	—
C20:0	2.9	1.1	1.3
C20:1	0.2	1.8	1.4
C20:2	—	1.9	1.0
C20:3	3.1	2.8	1.5
C20:4	6.0	6.5	2.0

may promote cholesterol ester accumulation in some foam cells in atherosclerosis.

Macrophages are the major site of lipid accumulation in Tangier disease (3). In spite of the virtual absence of high density lipoprotein, the lipoprotein class believed responsible for cholesterol mobilization from cells (29, 30) most cells in Tangier patients do not develop cholesterol ester-rich droplets. By regulating their uptake of low density lipoprotein by mechanisms such as those proposed by Goldstein and Brown (31), most cells may be able to maintain a normal cholesterol content. For example, the smooth muscle cells of the splenic artery in our patient showed no evidence of cholesterol ester accumulation. Macrophages, however, have an obligate intake of cholesterol because the material they phagocytose such as effete erythrocytes, other moribund cells, cellular debris, and bacteria, contains cholesterol. Thus, macrophages would be much more dependent on cholesterol removal to maintain a normal level of intracellular cholesterol. Werb and Cohn have shown that macrophages grown in culture and enriched with cholesterol do not normalize their cholesterol content in delipidated serum (lipoprotein free media) but will do so rapidly in the presence of lipoprotein containing serum (28). Thus, macrophages appear to require lipoprotein, presumably high density lipoproteins to remove cholesterol. Since cholesterol is insoluble in water, and the ability of the cells to solubilize free cholesterol in the membrane phospholipids is limited (7, 32) excess cholesterol is stored

as its ester in the liquid crystalline or liquid state. In this storage form, the cholesterol ester droplets do not appear to compromise the viability of cells, since no evidence of cell necrosis was found in this or previous studies (2, 3).

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