Cholestatriene and ergostatetraene as in vivo and in vitro membrane and lipoprotein probes

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Abstract The fluorescent cholesterol analogues, cholesta-5,7,9(11)triene-3-β-ol (I) and ergosta-5,7,9(11)-22-tetraene-3-β-ol (II), have been shown to be readily incorporated by various tissues and lipoproteins in rabbits maintained on diets supplemented with these fluorophores. Human erythrocytes and lipoproteins were also found to incorporate I and II in vitro under physiological conditions. The thermotropic behavior of the lipoproteins and erythrocyte membranes labeled with sterols I and II was evaluated using temperature-dependent fluorescence polarization and/or fluorescence intensity spectra. Erythrocyte ghosts, fluorescently labeled in vivo (rabbit) or in vitro (rabbit and human), were found to undergo a reversible thermally induced transition at 24 ± 2°C. A similar transition occurring at higher temperatures was also observed in fluorescently labeled human and rabbit LDL particles. Furthermore, the transition temperatures and relative microviscosities of the in vivo labeled rabbit LDL particles were found to be dependent upon the amount of sterol present in the rabbits' diet. No evidence of a similar thermotropic transition was observed in any of the HDL particles. These results are discussed in terms of a thermotropic reordering of cholesterol clusters existing in the erythrocyte membrane and of the cholesteryl ester core present within the low density lipoprotein particle.—**Bergeron, R. J., and J. Scott.** Cholestatriene and ergostatetraene as in vivo and in vitro membrane and lipoprotein probes. *J. Lipid Res.* 1982. **23:** 391–404

Supplementary key words cholesterol clusters • cholesteryl ester core • thermotropic transitions

In recent years, a great deal of effort has been focused on the nature of membranes and lipoproteins, and their interactions with each other (1, 2). Although model studies have provided substantial information about these systems, extrapolation of these findings to actual cell membranes and lipoproteins has proved difficult. These systems, by often omitting important components, e.g., proteins in the membrane models or certain of the lipids in the lipoprotein models, are, in many regards, too simplistic. However, the major problem encountered in membrane/lipoprotein studies is one of an experimental nature; spectroscopic monitoring of their components is difficult and extrinsic probes must often be introduced (3–6). Because of the usually non-biological nature of these probes and/or the manner of their incorporation, perturbation frequently occurs (7). Furthermore, even in the apparent absence of perturbations, the probes' disposition amongst the various lipid components is frequently not clear (8).

Recently, however, it was demonstrated that cholesta-5,7,9(11)triene-3-β-ol (I) and ergosta-5,7,9(11)-22-tetraene-3-β-ol (II) were readily incorporated into the bacterial membranes of *Tetrahymena pyriformis* and *Mycoplasma mycoides* when each was grown on a medium containing these compounds (9, 10). These fluorescent sterol probes, close structural analogues of cholesterol, were capable of totally replacing sterols thought to be essential to the growth of these organisms. This, coupled with their close structural relationship to 7-dehydrocholesterol, a biological precursor of cholesterol capable of replacing it within erythrocyte membranes and lipoproteins (11), suggested their use as sterol probes in animal membranes and lipoproteins.

In this study, we report the in vivo labeling of rabbit lipoproteins, red cell membranes, and hard tissues in addition to the in vitro labeling of human erythrocytes and lipoproteins with the fluorescent cholesterol analogues I or II. Once labeled, the thermotropic behavior of both the erythrocyte membranes and lipoproteins can easily be monitored by observing the fluorescence intensity or fluorescence polarization of compounds I or II as a function of temperature. Furthermore, by incubating labeled lipoproteins with unlabeled red cells, the transfer of the fluorescent sterol from lipoproteins to the red cell wall can be measured. The reverse reaction can be measured using labeled red blood cells and unlabeled lipoproteins. Our results demonstrate that these fluorescent sterols could have broad applicability in areas of biomed-

Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; HSA, human serum albumin; LDL, low density lipoproteins; HDL, high density lipoproteins; Amp B, Amphotericin B.

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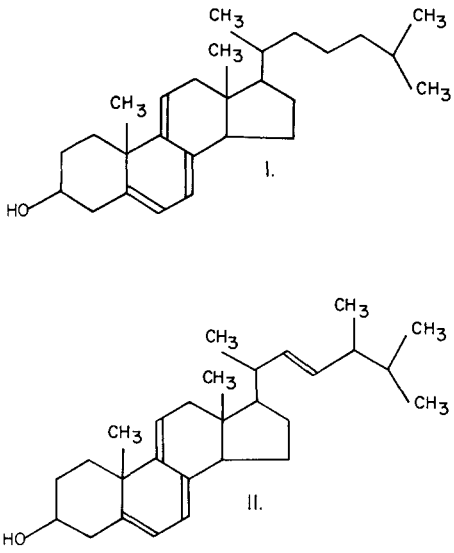

Fig. 1. Cholesta-5,7,9(11)-triene-3-β-ol (I) and ergosta-5,7,9(11)-22-tetraene-3-β-ol (II).

ical research which presently rely on radioactive labeling in order to report on cholesterol absorption, transport, and deposition (12, 13). Finally, these probes are shown to be non-perturbing and their physiological transport and delivery appear to be identical with that of cholesterol.

MATERIALS AND METHODS

Chemicals

All reagents were obtained from Aldrich Chemical Company. Water used in buffer preparation was glass-distilled. Heparinized Vacutainers and siliconized test tubes were obtained from Kimble. Digitonin, thimerosol, diphenylhexatriene, dipalmitoylphosphatidylcholine, and human serum albumin were obtained from Sigma Chemical Company. Amphotericin B was obtained from Squibb and Sons. Cholesterol-free rabbit diet was obtained from ICN Pharmaceuticals.

Synthesis of fluorescent cholesterol analogues

Cholesta-5,7,9(11)triene-3-β-ol (I) and ergosta-5,-7,9(11)-22-tetraene-3-β-ol (II) (**Fig. 1**) were synthesized according to previous procedures (14, 15). The final products were crystallized initially from methanol and then four times from acetone, mp 120–121°C (I) and 145–146°C (II), (literature 119–121°C and 145–147°C) (14, 15). The absorption spectra were identical with those reported earlier (10, 16) with maxima at 313, 327, and 342 nm. The degree of inflection at 297 nm was used to ascertain the degree of $\Delta^{5,7}$-contamination (15). Fluorescent spectra of both compounds were identical to those reported previously (17) with emission maxima occurring at 352, 374, and at 390 nm. Calculated masses for I and II are 382 and 394, respectively. Mass spectra taken of these two compounds showed molecular ions at 382 and 394 and none corresponding to the starting material, i.e., at 384 or 396.

Blood and animal tissues

Studies involving red blood cells employed human (6 ml) or rabbit blood (3 ml) drawn directly into 6-ml heparinized Vacutainers. Prior to use, all blood was centrifuged, the plasma and buffy coat were removed, and the cells were washed four times with 10-ml volumes of pH 7.4 isotonic buffer. This buffer consisted of 150 mM NaCl, 5 mM KCl, 3.5 mM Na_2HPO_4, 1.5 mM NaH_2PO_4, and 5 mM glucose. Cholesterol-depleted red blood cels were prepared by incubating washed red blood cells with cholesterol-depleted plasma for 12 hr at 37°C (18).

Other tissue samples excised from sacrificed animals including liver, spleen, kidney, brain, and lymphocytes were stored frozen or were suspended in the isotonic buffer supplemented with penicillin at 4°C.

Isolation of lipoprotein fractions

Plasma obtained from the centrifugation of whole blood samples was treated with an inhibitor solution (1:100 v/v) consisting of 49.4 mM thimerosol, 154 mM sodium azide, 26.9 mM EDTA, and trypsin inhibitor, and centrifuged for 20 hr at 108,000 *g* at 4°C in a Beckman ultracentrifuge. After the initial centrifugation, the upper layer was discarded and the lower layers were pooled. The density of the combined layers was adjusted to 1.063 g/ml using KBr and centrifuged. Following the second centrifugation, the LDL upper layer was drawn off and the density of the remaining bottom layers was adjusted to 1.21 g/ml with KBr. Following the final centrifugation, the uppermost layer, now containing the purified HDL fractions, was isolated and each lipoprotein fraction was dialyzed for 48 hr against two changes of pH 7.4 isotonic buffer at 4°C.

Tissue treatment prior to fluorescence analysis

Red blood cells, whether labeled in vivo (rabbit) or in vitro (human and rabbit) were converted to ghosts prior to measurement of membrane thermotropic behavior (19). Packed washed cells (0.2 to 1.0 ml) were lysed by suspending them in 40 ml of a 1:5 dilution of the pH 7.4 isotonic buffer at 4°C for 20 min. The lysed membranes were then centrifuged for 20 min at 15,000 rpm at 4°C. Resealing of these membranes was accomplished by resuspending the ghost pellet in 40 ml of pH 7.4

isotonic buffer and incubating at 37°C for 30 min. These membranes were then isolated by recentrifugation (15,000 rpm) at 4°C and resuspended in 40 ml of fresh isotonic buffer. This washing procedure was carried out four times to ensure that the final preparation was free of any fluorophore not bound to the membrane. Fluorescence spectra taken of hexane extracts of the final wash showed no analogue to be present. Following the last wash, the ghosts were suspended in 250 ml of pH 7.4 isotonic buffer ($0.4–2.0 \times 10^7 \pm 5\%$ ghost membranes/ml) and used immediately. Labeled and unlabeled red cells used in sterol exchange experiments were simply washed with isotonic buffer prior to use.

Other tissues from the treated animals were first washed extensively in pH 7.4 isotonic buffer. Then, small sections, approximately 1 g wet weight, of each of these tissues were homogenized in 20 ml of cold pH 7.4 isotonic buffer using a Potter-Elvejhem tissue homogenizer. The homogenized tissue was centrifuged, the buffer was decanted, and the tissue pellet was resuspended in fresh buffer (40 ml). This suspension was sonicated in a Cole-Parmer sonicator to disrupt membrane clumps and recentrifuged. After the washing procedure was carried out three times, the pellet was extracted with 10 ml of ethanol–acetone–ether 4:4:1. After sonicating and vortexing the extraction mixture, the pellet was isolated by centrifugation and the extracting solvents were removed. The extracts were placed in 12-ml siliconized test tubes and evaporated to dryness under N_2. Isotonic buffer (6 ml) was added and the tubes were sonicated briefly. The presence of the probe compound was confirmed by comparison of the fluorescence spectra of these lipid dispersions with that obtained from fluorophore-labeled dipalmitoyl phosphatidylcholine liposomes as prepared by others (10). No analyses were undertaken with these tissues to quantitate the amount of probe present relative to membrane-bound protein.

Spectroscopic studies

All UV absorption spectra were taken with a Beckman spectrophotometer, model 25. The fluorescence spectra were obtained using a Perkin-Elmer model 44-B MPF spectrofluorometer equipped with a temperature-controlled cuvette holder and Polaroid polarization filters. All spectra were uncorrected and taken at room temperature at pH 7.4 unless otherwise specified. In all experiments in which a pH other than 7.4 was required, the working pH of the stock membranes was adjusted using concentrated HCl or aqueous NaOH. Dilution effects due to this change in pH were found to be negligible. Experiments involving membranes utilized red blood cell ghosts prepared from whole blood previously labeled in vitro (human and rabbit) or in vivo (rabbit only) with one of the two fluorescent probes. Membrane samples were prepared from 0.2 ml to 1.0 ml of packed labeled erythrocytes. Following conversion to ghosts, the membranes were suspended in 250 ml of pH 7.4 isotonic buffer. These suspensions contained $0.4–2.0 \times 10^7 \pm 5\%$ ghost membranes/ml containing 0.3 nmol of probe (in vitro) to 280 nmol of probe/mg ghost protein (in vivo). Lipoprotein samples prepared by us contained 0.05 mg to 1.5 mg of probe/mg protein. Fluorescence emission spectra of these samples were obtained using an excitation wavelength between 310–330 nm while monitoring emission from 350–410 nm. The excitation and emission slit-widths were set at 6 nm and 5 nm, respectively. Using multiple spectra from the same labeled stock solution and similar unlabeled solutions, fluctuations in fluorescence emission intensity due to scattering were found to be ±2%. When fluorescence was measured as a function of temperature, the temperature was continuously monitored using a YSI tele-thermometer immersed in the sample cuvette. The reported precision for this probe is ±0.1°C. The cuvette temperature was controlled by a Lauda K-2/R circulating both. The range of temperatures evaluated using erythrocyte ghosts varied from 10 to 45°C and from 10 to 60°C for the lipoprotein samples. In order to evaluate the reversibility of any observed phenomena, temperature changes were undertaken in both directions. Temperatures greater than 45°C were not used in the membrane studies because of irreversible membrane protein denaturation above this temperature (20). Moisture condensation on the cuvette surface at low temperatures was eliminated by continuous flushing of the cuvette chamber with cold dry N_2.

Fluorescence polarization measurements were used to further evaluate the thermotropic behavior of the labeled lipoprotein classes as well as their relative microviscosities. Polarization values (P) and rotational anisotropy values (r) were obtained using the equations:

$$P = \frac{I_{\parallel} - cI_{\perp}}{I_{\parallel} + cI_{\perp}}$$

$$r = 2P/3\text{-}P$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities observed parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the beam of polarized excitation light. The term (c) is a correction for instrument polarization. The procedure for obtaining these values has been described in detail earlier (21).

Because the fluorescence intensities of the two probes decreased over time when exposed to the exciting UV light source, only one spectral data point was taken from each sample. Data points and ranges normally represent the spectra of at least three separate samples taken at each temperature and pH value.

In vivo incorporation of fluorescent cholesterol analogues

New Zealand rabbits were maintained from 3 days to 6 weeks on diets supplemented with one of the two fluorophores. The rabbits used in the membrane incorporation/thermotropic behavior studies normally received 0.1% to 0.5% (w/w) amounts of either fluorophore I or II, whereas those animals involved in the lipoprotein studies received 0.01% to 0.5% (w/w) amounts of only fluorophore I. In order to produce lipoprotein particles containing increasing amounts of sterol, some rabbits used in the lipoprotein studies also received varying amounts of cholesterol 0.1% to 3.0% w/w) in addition to the fluorophore in their diets. All control rabbits in both studies received fluorophore-free diets supplemented only with equivalent amounts of cholesterol. The experimental feed was prepared by adding the appropriate amounts of sterol dissolved in anhydrous diethyl ether to the correct amount of chow. After soaking for a short period the solvent was removed by subjecting the samples to high vacuum for 12 hr. Blood samples were drawn periodically from the long ear veins of the animals during the experimental feedings and analyzed fluorometrically to determine the amount of cholesterol analogue appearing in the erythrocyte membrane and plasma. At the end of the feeding periods, the animals were killed using an overdose of phenobarbital or by asphyxiation. The blood was removed via heart puncture and stored in heparinized tubes at 4°C. Other tissue types excised from the animals, i.e., brain, liver, kidney, spleen, and lymphocytes, were frozen immediately or were stored at 4°C in penicillin-treated pH 7.4 isotonic buffer.

In vitro incorporation of fluorescent cholesterol analogues by human and rabbit red blood cells

Both sterol probes were incorporated into washed human and rabbit red blood cells using 1% human serum albumin (HSA) in pH 7.4 isotonic buffer (22). Normally, 0.02 to 1.5 μmol of a sterol fluorophore was coated onto the inside of a 10-ml volumetric flask by first dissolving the steroid in 2 ml of ether and then removing the solvent by evaporation under N_2. The sterol residue was treated with 10 ml of a 1% HSA solution, and the vessel was immersed in a Cole-Parmer sonicator for five minutes and then vortexed briefly. Suspensions of erythrocytes were prepared (9 → 33% hematocrit) by combining 1 to 5 ml of packed, washed red blood cells with 10-ml volumes of the labeling solutions. This mixture, normally containing 18.0–54.0 nmol of fluorophore/mg protein, was rotated continuously at 37°C for periods of time ranging from 15 min to 24 hr. Following the incubation period, the fluorescently labeled cells were isolated by centrifugation, washed four times with 10-ml volumes of pH 7.4 isotonic buffer and stored at 4°C until used. All cells were used within 48 hr.

In vitro incorporation of fluorescent cholesterol analogues by lipoproteins

The in vitro incorporation of analogue I by human lipoproteins was undertaken in the absence and presence of serum esterases. Lipoproteins containing only the non-esterified form of the fluorophore were obtained by incubating purified samples of the lipoprotein (LDL or HDL) with washed red blood cells which had been labeled in vitro as previously described using fluorophore containing 1% HSA, i.e., in the absence of serum esterase activity. Normally, 5 ml of washed human red blood cells was fluorescently labeled by incubation with 10 ml of 1% human serum albumin in pH 7.4 isotonic buffer previously treated with 0.5 to 1.5 μmol of the fluorophore. This mixture, containing 18.0–54.0 nmol of fluorophore/mg ghost protein, was rotated continuously for 4 hr at 37°C, after which the fluorescently labeled cells were isolated by centrifugation. The labeled cells were then washed three times with 40 ml of isotonic buffer (pH 7.4) and incubated as above with purified LDL (0.87 mg protein/ml). After 4 hr, the fluorescently labeled LDL was isolated by centrifugational pelleting of the red blood cells.

Alternatively, lipoproteins containing both non-esterified fluorophore and biologically esterified fluorophore were obtained by incubating label-containing 1% HSA with whole blood, i.e., in the presence of serum esterase activity. Whole blood (15 ml) was incubated at 37°C with 10 ml of a 1% HSA solution containing 1.0 to 1.5 μmol of fluorophore. After 48 hr, the plasma was isolated by centrifugation and the lipoprotein fractions containing both esterified and non-esterified fluorophore were separated using ultracentrifugation as previously described.

Analysis of blood samples

Digitonin precipitation was used to quantitate the amount of fluorophore and total sterol present in the labeled erythrocyte membranes. This saponin has been shown to quantitatively precipitate sterols possessing a 3-β-hydroxyl group (23). Prior to its use, it was determined that excess digitonin did precipitate both fluorophores quantitatively and did not interfere with the colorimetric quantitation of total sterol as determined using the Liebermann-Burchard reagent (24). Samples of packed washed red blood cells (0.2 ml) labeled in vivo or in vitro were pipetted into 12-ml siliconized test tubes, hemolyzed with 0.4 ml of distilled water, and extracted with 10 ml of acetone–ethanol–ether 4:4:1. After vortexing and sonicating, the tubes were centrifuged and the supernatants were decanted. After reducing the sample

volume to ≈2 ml under N_2, the free sterol was isolated using a 2:1 addition of 1% digitonin solution. The digitonides were isolated by centifugation after 6–12 hr of incubation, washed three times with 1.0 ml of ether and stored at −20°C. The supernatants separated from these digitonides and the ether washings were combined and then saponified with methanolic KOH at 40°C for 30 min. After neutralizing and again reducing the volume of each sample, the solutions were treated with 2:1 volumes of the 1% digitonin reagent and allowed to precipitate as previously. Following this final precipitation, both digitonides were dissolved in 8 ml of glacial acetic acid and analyzed fluorometrically. The amounts of free and total fluorophore were then calculated using Beer's law plots derived from serial dilutions of the fluorophores (1×10^{-7} M to 1×10^{-5} M) in glacial acetic acid. Similarly, free and total sterol amounts were determined by treating the two digitonide groups with Liebermann-Burchard reagent and analyzing colorimetrically (34). The difference between the amounts of membrane sterol and fluorophore was used to estimate the amount of cholesterol present in the membranes. Protein determinations of erythrocyte ghosts employed the method of Lowry et al. (25). The number of erythrocytes or ghosts present in stock solutions was determined using a SC-2 Coulter counter equipped with hard copy readout.

Analysis of lipoprotein fractions

The amounts of free and esterified sterol probe incorporated by the purified LDL and HDL samples were also determined using digitonin precipitation. Samples of the lipoprotein 10–100 μl labeled in vivo or in vitro were pipetted into 12-ml siliconized test tubes and treated as above. Protein determinations were also as described (25).

Förster energy transfer between 1,6-diphenyl-1,3,5-hexatriene, Amphotericin B and the fluorescent probes I and II

The ability of the two cholesterol analogues to engage in Förster energy transfer (26, 27) while membrane-bound was investigated using 1,6-diphenyl-1,3,5-hexatriene (DPH), and Amphotericin B (Amp B), as energy acceptors. The UV absorption spectra of these two compounds are such that a large degree of overlap exists between their absorption spectra and the fluorescence emission spectra of analogues I and II. Therefore, under the correct conditions of distance and orientations between these acceptor compounds and the cholesterol analogues I or II, resonance energy transfer between the two should occur. These experiments were undertaken at room temperature using both lipid dispersions and erythrocyte membranes. The lipid dispersions were prepared using dipalmitoylphosphatidylcholine (1×10^{-5} M), cholesterol, and analogue I or II. The amounts of the individual lipids were adjusted such that the assay concentrations of cholesterol analogue varied from 9×10^{-8} to 2×10^{-6} M with the mole fractions of phospholipid and total sterol remaining equal. Stock solutions 1.5×10^{-3} M of Amp B in distilled water and DPH in tetrahydrofuran were prepared. Volumes of these two solutions (10 μl → 200 μl) were added to the lipid dispersions such that the acceptor concentrations (Amp B or DPH) in these samples varied from 2×10^{-8} to 5×10^{-6} M. These solutions were then incubated for periods up to 4 hr at 37°C with constant rotation of the sample. At different time intervals, samples were assayed fluorometrically by exciting at 325 nm while monitoring the emission between 370 nm to 550 nm. The quenching of the fluorescence emission spectra of the cholesterol analogues and the sensitized fluorescence emission of DPH and Amp B were then analyzed as a function of incubation time and acceptor concentrations. Studies identical to the above were also undertaken using erythrocyte membranes previously labeled in vitro with I or II (30.0 nmol probe/ml packed cells). Volumes of Amp B and DPH stock solutions used in these assays were such that the amount of these components incubated with the erythrocyte membranes varied from 8 nmol to 2 μmol/ml packed cells.

Förster energy transfer between erythrocyte membrane proteins and the cholesterol analogues

Red blood cells and ghosts, labeled in vitro (rabbit and human) or in vivo (rabbit only), were evaluated for any evidence of resonance energy transfer occurring between intrinsically fluorescent membrane-bound proteins and the fluorescent cholesterol analogues over the temperature range 4°C to 41°C. Washed red blood cells or ghosts containing one of the fluorescent probes, 0.3 nmol of probe/mg ghost protein (in vitro) to 280 nmol of probe/mg ghost protein (in vivo), were suspended in pH 7.4 isotonic buffer at a cell or ghost concentration of 0.4 to $2.0 \times 10^7 \pm 5\%$ cells or ghosts/ml. Control solutions were similarly prepared using red blood cells or ghosts that contained neither fluorophore. Fluorescence emission spectra for both the treated and control samples were taken using excitation wavelengths between 270 and 290 nm while monitoring the fluorescence emission between 300 and 430 nm. The fluorescent intensities of the emission spectra obtained for these two groups were subsequently normalized according to the number of cells/sample or mg of protein present/sample. The labeled samples were then evaluated relative to the unlabeled control samples for any evidence of fluorescence quenching of the membrane-bound protein fluorophores and/or sensitized fluorescence of the cholesterol analogues.

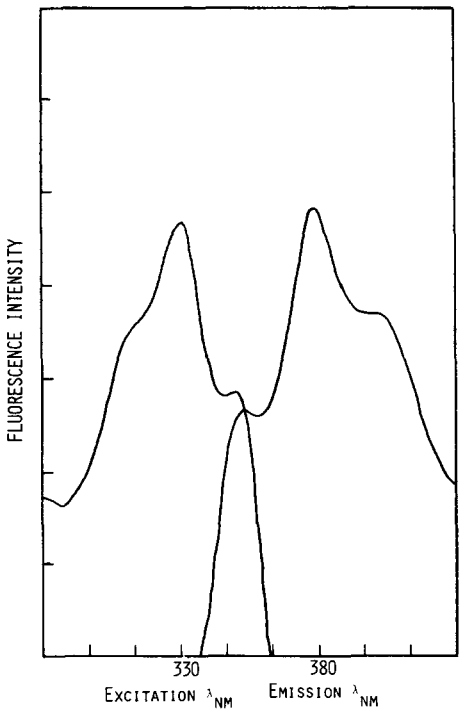

Fig. 2. The fluorescence excitation and emission spectra of rabbit ghost membranes ($\approx 6 \times 10^6$ ghosts/ml) containing 0.34 mg of cholesta-5,7,9(11)-triene-3-β-ol/ml packed cells in pH 7.4 isotonic buffer at room temperature.

RESULTS

Fluorescence characteristics of cholesterol analogues in membranes and lipoproteins

The UV absorption characteristics of whole red blood cells are such that the UV spectra of compounds I and II, when incorporated into these cells, are obscured. Additionally, because of the large amount of hemoglobin present in these cells, the fluorescence spectra of the two fluorophores, although visible, is strongly quenched. Therefore, all experiments dealing with erythrocyte membrane thermotropic behavior were undertaken using red blood cell ghosts rather than whole cells. **Fig. 2** shows the fluorescence emission and fluorescence excitation spectra of labeled rabbit ghosts. The fluorescence spectra of whole labeled lymphocytes, organic extracts of brain tissue homogenates, and the lipoprotein fractions, LDL and HDL, obtained from rabbits maintained on the diets, supplemented with the cholesterol analogue, are shown in **Fig. 3**. The spectra from all the labeled species are identical with a characteristic peak maximum at 374 nm and a shoulder at 390 nm.

In vivo and in vitro incorporation of fluorophores I and II

Red blood cells obtained from rabbits maintained on diets supplemented with fluorophore I or II (0.1–0.5%) were assayed for these compounds during the first days of feeding. Similar analyses were also undertaken of the human and rabbit red blood cells which were incubated in vitro with label containing 1% HSA solution. In both, the incorporation of these steroids by the red blood cells followed a normal absorption isotherm, with the in vivo incorporation method producing greater levels of incorporation relative to the in vitro method under the conditions described (**Fig. 4**). In practice, the amounts of probe incorporated in vivo varied with the composition of the diet (Fig. 4), the length of feedings (Fig. 4), and the experimental animal, whereas the amounts incorporated in vitro varied with incubation temperature, analogue concentrations, and length of incubation (**Fig. 5** and **Fig. 6**). Analyses of the labeled membranes showed 2.7–280 nmol of probe/mg ghost protein (in vivo) and 0.3–8.8 nmol of probe/mg ghost protein (in vitro) to be incorporated under the conditions described, with the amount of compound I incorporated exceeding that of compound II by 20 ± 5%. Furthermore, red blood cells pretreated with cholesterol-depleted plasma prior to incubation with the fluorophore were found to incorporate greater amounts of the fluorophore than the non-depleted

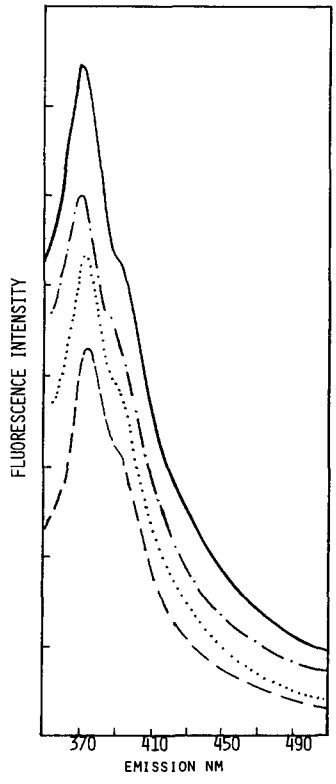

Fig. 3. Fluorescence emission spectra (λ_{exc} 320) of rabbit lymphocytes (——), brain extract (–·–), low density lipoprotein (-----), and high density lipoprotein (–––) labeled in vivo with ergosta-5,7,9(11)-22-tetraene-3-β-ol (II). No relationship is implied between the fluorescence emission intensities presented and the amount of cholesterol analogue present in each of the above biological samples.

cells. Because of the relatively large amounts of the two fluorophores incorporated into the treated rabbit erythrocytes in vivo, additional analyses were undertaken over 10-day feeding periods in order to evaluate the changes occurring in the total amount of sterol present within these treated membranes, relative to control animals fed a similar diet. Those rabbits receiving 0.5% (w/w) amounts of fluorophore I were found to have increased the total sterol content of their erythrocyte membranes from 132 ± 13 mg/100 ml to 192 ± 20 mg/100 ml with the mole ratio of fluorophore/cholesterol changing from 3.4×10^{-4} (4 hr) to 2.6×10^{-1} (10 days). During the same period, the level of sterol present in the erythrocytes from rabbits belonging to the control group (0.5% w/w cholesterol only) changed from 116 ± 12 mg/100 ml to 202 ± 20 mg/100 ml. Similar analyses of red blood cells labeled in vitro demonstrated that no overall change in sterol content occurred in these cells during the incubation within the sensitivity of our analyses. Surprisingly, digitonin analysis of the in vivo labeled blood cells appeared to indicate that ≈10 ± 5% of the incorporated sterol appeared to be present in the esterified form.

Purified lipoprotein particles labeled in vivo (rabbit) and in vitro (human) were also analyzed to determine the amounts of free and esterified fluorophore I incorporated. The total uptake of fluorophore I by LDL varied from 0.5 mg probe/mg protein (in vitro) to 1.45 mg probe/mg protein (in vivo), whereas the amounts incorporated by HDL varied from 0.06–0.11 mg probe/mg protein, respectively. Factors found to affect the actual amounts of I incorporated by these particles included: the method of incorporation (in vivo or in vitro), the amount of fluorophore present in the diet or the incubation mixture (Table 1), the length of the feeding or incubation period (Fig. 5), the class of lipoprotein analyzed, and the experimental animal. When the probe was incorporated by the lipoprotein in vivo, a substantial amount was absorbed as the sterol ester (34–70%) because of LCAT activity present within the animal. Similar levels of esterified fluorophore were also incorporated in vitro (≈60%) using incubations that contained serum esterases. However, it was also possible to incorporate only the non-esterified fluorophore in vitro using incu-

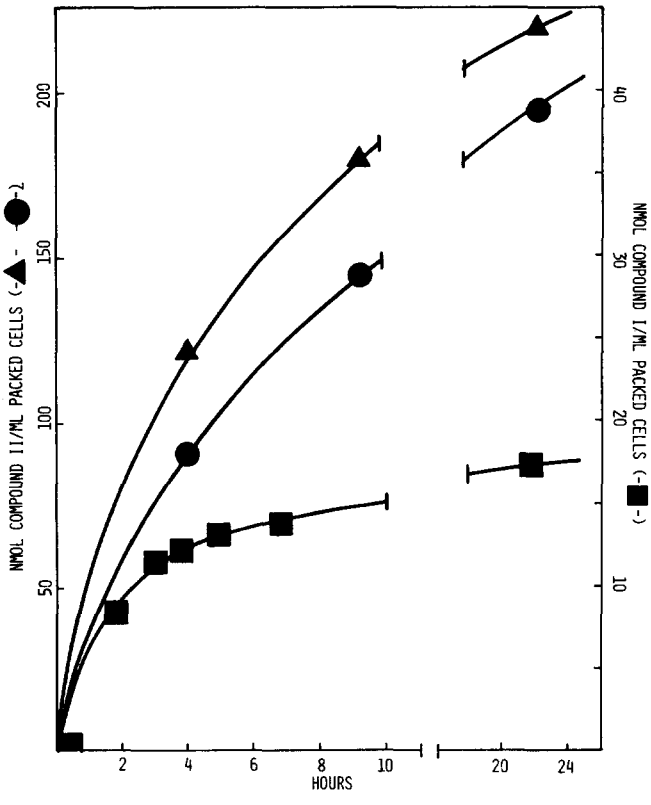

Fig. 4. Erythrocyte absorption of cholesterol analogues over 24 hours. Rabbits fed 0.5% w/w (—▲—) and 0.1% w/w (—●—) diets of ergosta-5,7,9(11)-22-tetraene-3-β-ol. Human erythrocytes incubated with 27 nmole of cholesta-5,7,9(11)-triene-3-β-ol/ml packed cells (—■—) at 37°C. Spectra were obtained at room temperature with λ_{ex} 320 nm and λ_{em} 374 nm.

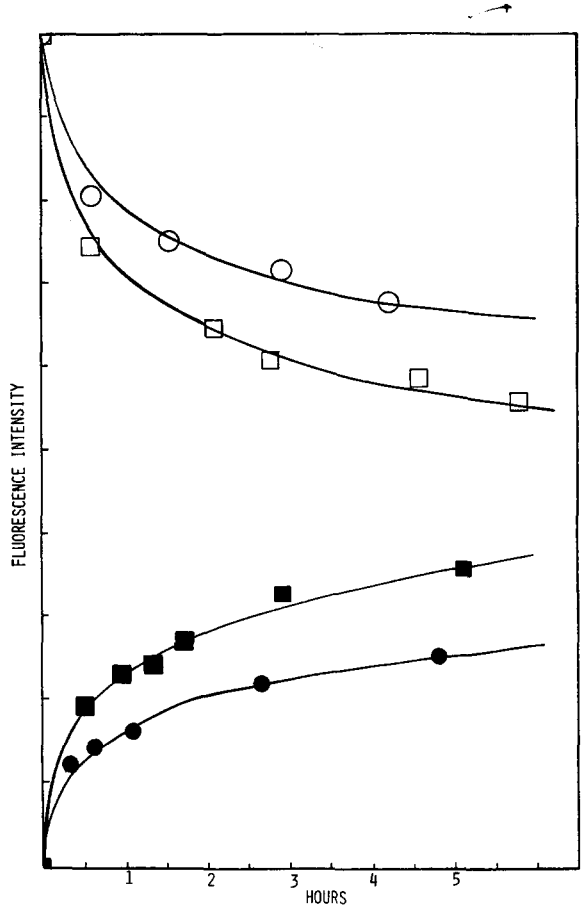

Fig. 5. Interaction of unlabeled human whole red blood cells with cholesta-5,7,9-(11)-triene-3-β-ol labeled LDL (1.06 μmol/mg protein) at 0°C (—○—) and 40°C (—□—) and purified unlabeled LDL with cholesta-5,7,9(11)-triene-3-β-ol labeled human red blood cells at (—●—) 0°C and 40°C (—■—). Spectra were obtained at room temperature with λ_{ex} 320 nm and λ_{em} 374 nm by following the change in fluorescence of the LDL isolated subsequent to the incubations.

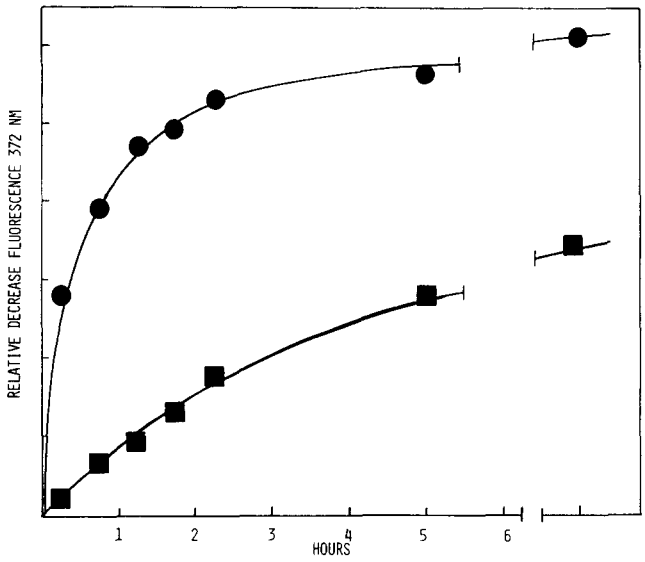

Fig. 6. Interaction of unlabeled whole red blood cells (200 μl packed cells/100 ml buffer) with 100 μl of cholesta-5,7,9(11)-triene-3-β-ol labeled LDL (1.06 μmol/mg protein) (—●—) and 200 μl of labeled HDL (0.18 μmol/mg protein (—■—) over 24-hr period at 37°C. Spectra of the incubation mixtures were recorded at room temperature in pH 7.4 isotonic buffer with λ_{ex} 320 and λ_{em} 374.

bation conditions in which no esterase activity was present.

In vitro exchange of fluorophore I between whole red blood cells and the lipoproteins LDL and HDL

Using both red blood cells and purified samples of LDL and HDL labeled in vivo (rabbit) and in vitro (human), the process of sterol exchange between these species was investigated. Because of the red blood cells' ability to quench the fluorescence emission of the fluorescent sterols, it was possible to follow the interaction of labeled lipoproteins with whole red blood cells. Purified samples of labeled LDL (1.06 μmol/mg protein) and labeled HDL (0.18 μmol/mg protein) were incubated at 4–40°C with washed unlabeled erythrocytes. The interaction between these red blood cells and the lipoproteins was then measured by the observed quenching of the label's fluorescence emission over time. The results at 30°C are given in Fig. 6. Fluorescent analysis of the hexane extract of these red blood cells subsequent to incubation and repeated washings demonstrated that transfer of the fluorescent probe to the erythrocyte was occurring. Furthermore, it has been shown that fluorophore I and its oleate esters can quench intrinsic lipoprotein fluorescence through the process of Förster resonance energy transfer (16). Analysis of the lipoproteins isolated subsequent to the above incubations would be expected to show decreased levels of probe fluorescence and increased levels of protein fluorescence. This was found to be the case, i.e., probe fluorescence decreased 30 to 40% in LDL samples and 15 to 25% in HDL samples whereas protein fluorescence increased 210 to 420% and 164 to 175%, respectively.

Alternatively, when red blood cells labeled in vitro using 1% HSA were incubated with lipoproteins, transfer of the nonesterified probe to the lipoprotein occurred. Uptake of the fluorophore by the purified lipoproteins followed a normal absorption curve with the amount incorporated being temperature-dependent (Fig. 5). Monitoring of the exchange process in this direction was readily accomplished by merely following the increased probe fluorescence in the lipoprotein samples following centrifugational pelleting of the red blood cells.

Thermotropic behavior of in vitro (human) and in vivo (rabbit) labeled lipoproteins

Human lipoproteins (LDL and HDL) were labeled in vitro such that fluorophore I was incorporated by these particles in both its nonesterified and/or its esterified forms. In those LDL samples containing only the nonesterified form of the cholesterol analogue, and in all of the HDL samples, no discontinuities were observed in any of the temperature-dependent fluorescence intensity plots (**Fig. 7**). However, LDL particles labeled in vitro such that esterified fluorophore was present underwent a clear thermotropic transition at 30 ± 2°C (Fig. 7). In order to analyze the thermotropic behavior and relative microviscosities of purified LDL samples obtained from rabbits maintained for 2 weeks on diets containing 0.01 to 0.1% fluorophore I and 0.1 to 3% cholesterol, both

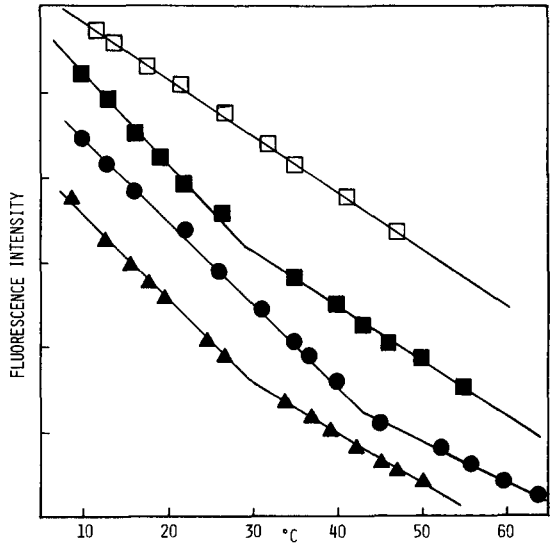

Fig. 7. Plots of fluorescence intensity vs. temperature for LDL labeled in vivo (rabbit) and in vitro (human) with cholesta-5,7,9(11)-triene-3-β-ol. Fluorescence spectra of rabbit LDL from animals maintained on 3.1% (—●—) and 0.01% (—▲—) sterol diets. Human LDL labeled in vitro with only the nonesterified fluorophore (—□—) or with both the nonesterified and esterified fluorophore (—■—).

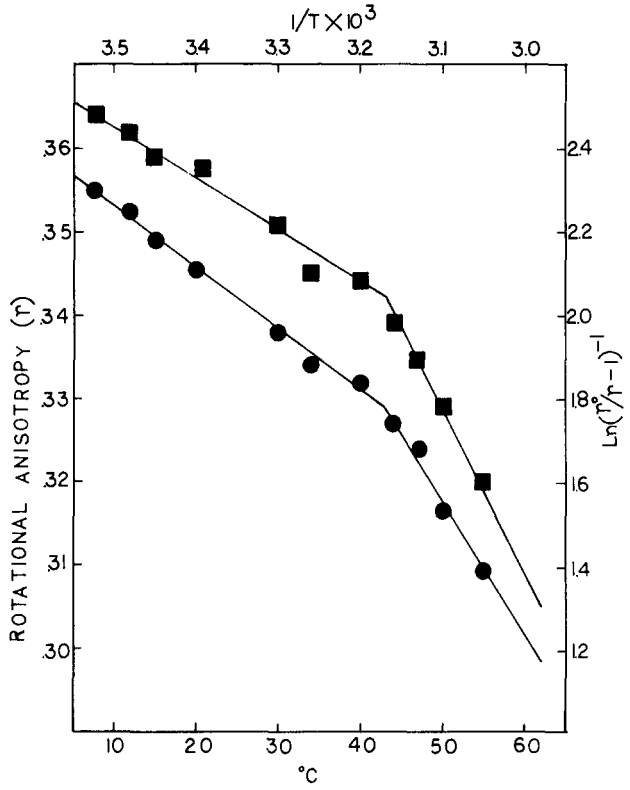

Fig. 8. The thermal transition temperature and relative apparent microviscosity of LDL labeled with cholesta-5,7,9(11)-triene-3-β-ol as determined from a plot of the fluorescence polarization parameters, r vs. temperature (—■—) and L_n $(r_0/r - 1)^{-1}$ vs. 1/T (—●—). Both spectra were obtained using labeled LDL from rabbits maintained on the 3.1% sterol enriched diet.

temperature-dependent fluorescence intensity and fluorescence polarization spectra were taken. Our results clearly demonstrated that a thermotropic transition occurred in these lipoproteins that was visible fluorometrically using either fluorescence intensity (Fig. 7) or fluorescence polarization spectra (**Fig. 8**). Furthermore, the temperature at which this transition occurred and the relative apparent microviscosity of the labeled LDL particles were found to be dependent upon the amount of sterol present in the diets (**Table 1**). The polarization term $(r_0/r - 1)^{-1}$ has previously been shown to provide a means for relating microviscosities in systems containing the same fluorescent label (21) and for demonstrating the presence of thermotropic discontinuities in lipid systems (28, 29). It is surprising that similar fluorescent studies involving the thermotropic behavior of LDL using DPH have failed to note the thermotropic transition observed by us and others (30, 31) in LDL. It must be assumed, therefore, that DPH, like the free sterol form of cholestatriene, does not partition into the cholesteryl ester core or that it partitions selectively into only the acyl chains of these esters (32). The applicability of the thermotropic data derived using DPH as a probe would, therefore, appear to be of limited value within these systems.

Temperature perturbation of labeled erythrocyte ghosts

The fluorescence emission spectra of erythrocyte ghosts, labeled in vivo (rabbit) or in vitro (human or rabbit) with either fluorescent cholesterol analogue display a reversible temperature-induced discontinuity when the temperature is varied in either direction over the range 0 to 45°C, **Fig. 9**. Plots of fluorescence emission intensity vs. temperature for both human and rabbit erythrocytes show a thermotropic break to occur in these labeled membranes between 22 and 26°C (Fig. 9). The position of this break was found to be independent of the fluorophore used (I or II), the method of incorporation (in vivo or in vitro), the type of red blood cell (human or rabbit), the direction of temperature change, and hydrogen ion concentration between pH 6 and pH 8. Furthermore, the cells labeled in vitro that had been pretreated in order to deplete some of the membrane cholesterol (17 ± 5%) were also found to exhibit the same thermotropic discontinuity. The effect of temperature on lipid dispersions prepared from lipid extracts (33) of these labeled erythrocyte membranes was also investigated. In contrast to the behavior by the labeled ghosts, no temperature-induced discontinuities were encountered in any of the protein-free lipid dispersions (Fig. 9) prepared from these ghosts.

Resonance energy transfer experiments

When either lipid dispersions or erythrocyte ghosts labeled in vitro with compounds I or II were titrated with DPH or Amp B, some fluorescence energy quench-

TABLE 1. Phase transition temperatures and relative microviscosities of in vivo labeled rabbit LDL (cholesta-5,7,9(11)-triene-3-β-ol) from rabbits maintained on cholesterol-enriched diets (0.1%–3.0% w/w).

Diet % Sterol	Mg Probe / Mg Protein	Mg Steroid / Mg Protein	Transition Temperature	$\text{Ln}\left(\frac{r_0}{r} - 1\right)^{-1}$ @ 25°C
3.1	1.40 ± 0.07	7.2 ± 0.4	43 ± 2	2.04
1.1	1.45 ± 0.08	5.3 ± 0.3	38 ± 2	1.50
0.2	1.31 ± 0.07	2.6 ± 0.1	35 ± 2	1.13
0.01	1.01 ± 0.05	1.4 ± 0.1	27 ± 2	0.23

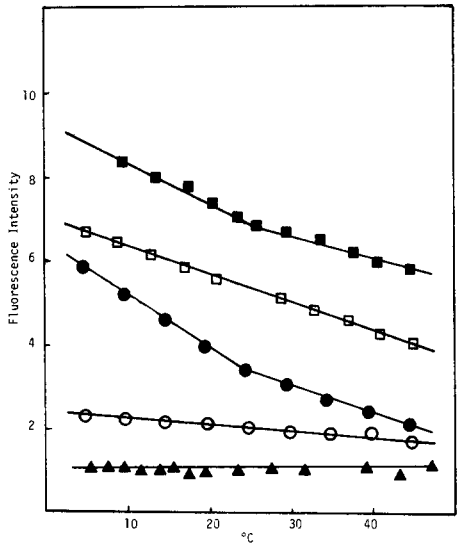

Fig. 9. The thermotropic behavior of rabbit (—■—) erythrocyte ghosts labeled in vivo with cholesta-5,7,9(11)-triene-3-β-ol and of human erythrocyte ghosts unlabeled (—▲—) and labeled (—●—) with ergosta-5,7,9(11)-22-tetraene-3-β-ol. The ratio of analogue to total membrane sterol in the labeled ghosts was 8.5×10^{-4} and 4.7×10^{-3}, respectively. Lipid dispersions prepared from the total lipid extracts of labeled erythrocytes, rabbit (—□—) and human (—○—).

ing of the sterols was observed. The fluorescence emission of I and II at 374 and 390 nm was quenched with concurrent increase in the emission intensity of DPH between 430 and 500 nm. Similarly, Amp B quenched the fluorescence emission of liposome and ghost-bound I and II while its emission at 469–490 nm was enhanced. At the highest concentration of Amp B (5.0 μM) used, the fluorescence intensity of the fluorescent analogue was reduced to approximately half of its non-quenched level in both the lipid dispersions and the erythrocyte ghosts. Although quenching of compound II by Amp B was observed in the past, it was not attributed to resonance energy transfer (9). The maximum absorption for Amp B in lipid dispersions was assumed to be the same as in aqueous solution, i.e., 329 nm (34). In fact, it has been demonstrated that in ethanolic solutions (35), lecithin/cholesterol dispersions, and erythrocyte membranes (34) the UV absorption maximum is significantly red-shifted to $\approx$370 nm. Therefore, rather than the wavelengths of maximum absorption for the fluorescent sterols and Amp B being the same, the absorption spectra of Amp B is instead shifted to wavelengths that strongly overlap the emission band of the fluorescing sterols. Similar quenching levels were also observed in DPH (0.5 μM)-titrated lipid dispersions, i.e., 57% at a donor/acceptor (D/A) ratio of 0.3; however, in labeled erythrocyte ghosts, only 17% quenching was observed at this same D/A ratio. The fact that only very low concentrations, normally less than 1×10^{-6} M, of either DPH or Amp B were used in the incubations and that the degree of quenching was dependent upon the partitioning of these species into the membrane, makes quenching by trivial reabsorption seem unlikely.

Finally, resonance energy transfer experiments were undertaken using in vivo (rabbit) and in vitro (human) labeled erythrocytes. The fluorescence emission spectra of whole red blood cells and ghosts display a broad tryptophan peak with a maxima at 330 nm. This emission band corresponds closely to the absorption maxima for both cholesterol analogues (327 nm). Under the correct conditions of distance and orientation between this donor/acceptor pair, energy transfer should occur. In fact, resonance energy transfer between compound I and its oleate ester with native tryptophan residues within the lipoproteins LDL and HDL has been demonstrated earlier (16). However, in neither the whole red blood cells nor ghosts, whether labeled in vivo (human) or in vitro (rabbit and human), with I or II, was evidence of protein fluorescence quenching or sensitized fluorescence encountered. Additional experiments undertaken at 4 and 41°C using in vitro labeled human ghosts also failed to provide any evidence for energy transfer between membrane proteins and the fluorescent sterols. These results suggest that the distances and/or orientations existing between the membrane proteins and the fluorescent sterols were such that energy transfer as described by Förster was not possible (28, 27).

DISCUSSION

The fluorescent sterols, cholesta-5,7,9(11)triene-3-β-ol and ergosta-5,7,9(11)-22-tetraene-3-β-ol, have been incorporated in vitro in human lipoproteins and erythrocytes and in vivo in rabbit lipoproteins, red cells, and hard tissues. Once incorporated, these probes facilitate the measurement of a number of physicochemical processes. The thermotropic behavior of both red cell membranes and lipoproteins as well as the exchange of sterols between them can be monitored easily.

Rabbits maintained on diets supplemented with the fluorescent sterols I or II incorporated these probes into a variety of tissues and lipoprotein fractions which are known to contain large amounts of cholesterol, i.e., spleen, kidney, liver erythrocytes, brain, LDL, and HDL. Erythrocyte absorption isotherms for rabbits maintained on 0.5% (w/w) and 0.1% (w/w) diets of compounds I and II demonstrated the expected behavior, i.e., increased dietary levels resulted in an increase in red cell absorption (Fig. 4). The absorption behavior of compounds I and II in erythrocytes was similar although at identical dietary levels 20 $\pm$ 5% more of compound I was absorbed over the same time period. However, in vitro HSA-loading of red cells with both probes showed less of a difference in total absorption. Compound I was ab-

sorbed 10 ± 5% more effectively. As demonstrated, both methods incorporate sufficient probe for the measurement of membrane thermotropic behavior; however, in vitro incorporation is less cumbersome. The levels of probe incorporated in vivo into the rabbit red cell membranes, although large, are not surprising in view of the hypercholesteremic-like diet and the length of feeding. Similar studies have amply demonstrated the rabbits' ability to incorporate large quantities of sterol into its erythrocytes, e.g., 500% increases, when maintained on diets of this type (36). Furthermore, it has been demonstrated that 7-dehydrocholesterol, the immediate structural precursor of fluorophore I, can replace up to 75% of rat erythrocyte cholesterol in vivo (37), 16% in vitro (38) and apparently 100% of human LDL cholesterol in vitro (37).

That both LDL and HDL exchanged compound I with whole red blood cells was clearly demonstrated by in vitro experiments (Figs. 5, 6). When red cells were first labeled with the probe and then incubated with unlabeled HDL or LDL and the lipoproteins were isolated, they were found to contain the probe. Conversely, when labeled lipoproteins were incubated with unlabeled red cells and the erythrocytes were isolated, they were found to contain compound I. These findings not only verified the mode of probe transport in vivo but also provided us with a vehicle for selectively adding or removing free sterol fluorophore from the lipoprotein surface.

This lipoprotein/red cell membrane interaction is also consistent with the cholesteryl ester levels we observed in the red cells when they were labeled in vivo. Analysis of the total fluorophore concentration in the erythrocyte membrane revealed that 10 ± 5% was in the esterified form. Although other workers have also reported the presence of cholesteryl ester in the red cells[2] (39), it is unclear whether or not the sterol itself is actually bound to the membrane or if it is bound as a lipoprotein complex (40). The presence of some bound lipoproten even after repeated washings would not be unreasonable in view of how the cholesterol is delivered to the membrane and the number of available lipoprotein receptors which had been reported by others (41, 42). However, the presence of any bound lipoprotein is irrelevant in terms of our interest in using the probes for evaluating the thermotropic behavior of the red cell membrane. Regardless of whether or not the red cells are labeled in vivo (rabbit) or in vitro (rabbit and human) with these probes, the membrane thermotropic behavior remains the same. Both human and rabbit ghosts show a clear discontinuity at 24° ± 2°C (Fig. 9). Moreover, when the total lipid extracts of these labeled erythrocytes are reconstituted as protein-free lipid dispersions, the inflections are absent (Fig. 9). Recently, other investigators have also demonstrated temperature-induced transitions in erythrocyte membranes in this same temperature range using such varied techniques as enzyme activity (24°C) (43), viscosity changes (19°C) (44), excimer formation (23°C) (45), and Raman spectroscopy (19°C) (46).

In order to evaluate the possible effects of increased levels of probe on the thermotropic behavior of the in vivo labeled erythrocytes, temperature/fluorescence intensity profiles were undertaken from 4 hr to 10 days after the initial fluorophore feeding (0.5% w/w). During this period, the analogue content of the fluorophore-treated rabbits' erythrocytes increased from 2.9 nmol/ml packed cells to 941 nmol/ml packed cells and the total sterol content increased to a level comparable to that present in similarly dieted (0.5% cholesterol w/w) control rabbits, i.e., 192 ± 19 mg/100 ml cells vs. 202 ± 20 mg/100 cells. However, even though the levels of cholesterol analogue relative to native cholesterol increased from 3.4×10^{-4} to 2.6×10^{-1} mole% and the total level of membrane sterol increased by nearly 45 ± 5% over the ten-day feeding, no change was seen in the position of the observed thermotropic discontinuity in any of these samples within the sensitivity range of our analyses. Furthermore, when erythrocytes (17% cholesterol depleted) were labeled with 1.52 mg of analogue I/100 ml cells, the inflection temperature again remained the same.

A possible explanation for the thermotropic transition we observed could be the presence of cholesterol clusters existing within the plane of the erythrocyte membrane. Recently, other evidence has been presented that suggests the existence of cholesterol clusters within the erythrocyte (47–52). It has been postulated that these sterol clusters are a direct result of cholesterol's exclusion from the lipid domains immediately surrounding integral proteins imbedded in the membrane (53). If domains in the erythrocyte's membrane are, in fact, comprised of protein-induced cholesterol clusters, they could be expected to demonstrate the kind of temperature discontinuity we observed and fluorescence energy transfer between the membrane proteins and sterol probes would be expected to be minimal. Moreover, in the absence of membrane proteins, i.e., in lipid dispersions prepared from protein-free erythrocyte lipids, the discontinuity would be expected to disappear. Although all of our thermotropic observations are, in fact, consistent with the cluster picture, it must be pointed out that the absence of energy transfer between membrane protein and the fluorescent sterols could also be attributed to improper donor/acceptor orientation. Unlike the red cell membranes we observed, the thermotropic behavior of lipoprotein particles labeled with fluorophore I was seen to be very sensitive to sterol concentration ranges.

[2] Harmony, J. K. Personal communication.

The lipoproteins LDL and HDL were labeled in vivo (rabbit) and in vitro (human) with compound I. As in the case of the labeled erythrocyte ghosts, the thermotropic behavior of these labeled particles was then evaluated using temperature-dependent fluorescence intensity spectra (Fig. 7). In addition, the rabbit lipoproteins were also studied using fluorescence polarization techniques (Fig. 8). Both methods clearly demonstrate that both human and rabbit LDL particles undergo a thermotropic transition whereas their HDL particles do not. Furthermore, it was found that in rabbits, both the phase change temperature and the relative microviscosity of the labeled LDL were dependent upon the diet the animals sustained. As the cholesterol in their diet was increased, the transition temperature and the apparent relative microviscosity of the low density lipoprotein particle also increased. Current evidence suggests that the thermotropic phase changed exhibited by LDL is produced by the thermal reordering of cholesterol esters located within the core of the lipoprotein particle (30, 31). Our results provide further evidence that a thermal reordering of the sterol esters was, in fact, responsible for this phenomenon. When human LDL was labeled in vitro with only the nonesterified form of the fluorophore, through exchange with in vitro labeled red blood cells, no phase changes were seen. However, when LDL particles were labeled in vivo (rabbit) or in vitro (human) in the presence of serum esterases so that both the nonesterified and esterified forms of the probe were incorporated by these particles, a clear thermotropic transition was observed. The increased temperature at which this transition occurred and the increased microviscosity observed in LDL samples obtained from hypercholesteremic rabbits are probably a result of improved cholesterol packing in the steroid-enriched core of these particles. Furthermore, based upon the thermotropic behavior exhibited by the in vivo labeled rabbit LDL, it would appear that if the esterified fluorophore which was reported to be present in the rabbit erythrocyte membrane was a component of a lipoprotein particle, it was not an LDL particle since no thermotropic transitions were observed in any of the ghost membrane preparations at the higher temperatures exhibited by the hypercholesterolemic rabbit LDL. Although we have been able to verify previously reported thermotropic changes in LDL using our probes (30, 31), other fluorescent studies utilizing DPH-labeled LDL have not (54, 55). Evidently, DPH partitions only into the outer shell of the LDL particle, analogous to the nonesterified form of cholestatriene, and/or intercalates solely between the alkyl chains of the core lipids rather than between the steroid nuclei. Finally, we wonder whether or not the workers who employed DPH to study the thermotropic properties of the red cell membranes (56, 57) failed to see any thermotropic discontinuities in these membranes for simialr reasons, i.e., because DPH did not partition into the cholesterol clusters. Indeed, the disparity we observed between DPH quenching of the sterol fluorophores located in lipid dispersions vs. erythrocyte ghosts could, in fact, be related to this.

CONCLUSION

The fluorescent probes cholesta-5,7,9(11)triene-3-β-ol and ergosta-5,7,9(11)-22-tetraene-3-β-ol are easily adsorbed into red blood cell membranes and lipoproteins. This labeling can be effected either in vivo or in vitro depending on what observations the investigator wishes to make.

Using these probes, we have generated evidence for cholesterol clustering in red cell membranes, in addition to providing more support for the theory that thermally-induced phase changes in LDL particles are associated with its central ester core. We have also cast further doubt on the viability of diphenylhexatriene as a probe for the evaluation of the physical properties of lipids in biologically condensed phases rich in cholesterol. Finally, we wish to point out that, because of their fluorescence, these cholesterol analogues can be used with greater facility than their radiolabeled counterparts for the measurement of the physicochemical properties of lipids in biological systems. Their transport and delivery is identical with that of cholesterol; they are LCAT substrates and their tissue distribution seems to be as ubiquitous as that of cholesterol. We are currently investigating the use of these probes in the evaluation of the membrane properties of other cell types.

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