

Fluorescent Low Density Lipoprotein for Observation of Dynamics of Individual Receptor Complexes on Cultured Human Fibroblasts

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ABSTRACT The visible wavelength excited fluorophore 3,3'-dioctadecylindocarbocyanine iodide (dil[3]) was incorporated into human low density lipoprotein (LDL) to form the highly fluorescent LDL derivative dil(3)-LDL. Dil(3)-LDL binds to normal human fibroblasts and to human fibroblasts defective in LDL receptor internalization but does not bind to LDL receptor-negative human fibroblasts at 4°C or 37°C. It is internalized rapidly at 37°C by normal fibroblasts and depresses the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) in a manner similar to that of LDL. It is prevented from binding to the LDL receptor by an excess of unlabeled LDL or by heparin sulfate. Identical distributions of dil(3)-LDL are observed on cells by either indirect immunofluorescence with fluorescein-labeled antibody or directly by dil(3) fluorescence. Upwards of 45 molecules of dil(3) are incorporated per molecule of LDL without affecting binding to the receptor. This labeling renders individual molecules visible by their fluorescence and enables the derivative to be used in dynamic studies of LDL-receptor motion on living fibroblasts by standard fluorescence techniques at low LDL receptor density. Observations with this derivative indicate that the LDL-receptor complex is immobilized on the surface of human fibroblasts but, when free of this linkage, undergoes a Brownian motion consistent with theory.

The means by which cells communicate information about ligand binding is the subject of intense investigation (1-5). Some of the insight gained in this area derives from study of the receptor system for low density lipoprotein (LDL) (6, 7). The LDL molecule is the predominant carrier of plasma cholesterol (6, 8, 9). Due to cholesterol's importance in the study of atherosclerosis and heart disease (6, 10), the genetics of certain diseases of cholesterol regulation are well known. In particular, diseases of cholesterol metabolism and synthesis have been associated with the LDL receptor (11-14). One human cell type, GM-2408A or J.D., has been shown to have a functional LDL receptor that binds LDL but is unable to effect its internalization (13, 15, 16). Another human cell line, GM-2000, lacks LDL receptors. In both conditions, the patients manifest elevated serum cholesterol levels and their cells are unable to regulate properly the enzymes involved in cholesterol synthesis (1, 6).

The LDL-receptor system shares various features common

to other hormone receptor systems (1, 2, 4, 5). LDL bound at 37°C is rapidly internalized by the cell in specific membrane areas called coated pits (17-19). Normal human fibroblasts contain preaggregated LDL receptor in coated pits (17, 18). Whether a receptor not located in a coated region can participate in signal transduction may depend on its ability to move from a noncoated to a coated area. This condition may apply to receptor systems other than the one for LDL (3).

Interested in the relationship of receptor motion to signal transduction, we chose to study this relationship in the LDL receptor system for the following reasons. Many aspects of the LDL system subsequent to receptor-ligand internalization have been characterized (1, 6). Cell mutants exist which are unable to properly regulate cholesterol synthesis due to defects in the LDL-receptor system, and the genetics of these defects has been studied (1, 6, 11-14). The LDL receptor system contains features characteristic of other hormone systems (1, 2, 4). Most important of all, the LDL molecule, because of its size and

structure, lends itself to multiple labeling by fluorophore while retaining its biological activity (20, 21). This last point will be discussed in detail below.

In its natural state, LDL is a large, spherical molecular complex ($M_r = 3 \times 10^6$, diameter = 240 Å) consisting of protein, phospholipid, triglyceride, cholesterol, and cholesterol ester (9, 22). Its exact structure is unknown, though differing models place phospholipids and its major protein component, apo B, at the surface of the sphere, with the cholesterol esters filling the interior (23, 24). A typical LDL may contain 750 phospholipid and 1,300 cholesterol ester molecules (24). The particular molecules that form the complex allow modification of LDL in several ways. Kreiger et al. (21) showed that the esters could be replaced with exogenous UV-excited fluorophores. The distribution of this fluorescent LDL derivative has been studied on normal fibroblasts (25). This derivative binds in a pattern similar to that of unsubstituted LDL. LDL in which the cholesterol ester has been removed but not replaced (26) also binds normally to cells but does not suppress the activity of the microsomal enzyme 3-hydroxy-3-methylglutaryl coenzyme-A reductase (HMG-CoA reductase). This enzyme regulates synthesis of endogenous cell cholesterol (27, 28). Lipid-soluble probes such as perylene can be incorporated into the lipid shell of LDL (29) by simple incubation. In the case of perylene there is probe exchange with the surrounding medium (29).

We report the incorporation of the lipid analog 3,3'-dioctadecylindolcarbocyanine (dil[3]) into human LDL to form the intensely fluorescent, visible wavelength-excited derivative dil(3)-LDL. The activity and binding specificity of dil(3)-LDL are demonstrated in the following ways. We show that dil(3)-LDL retains antigenic properties of LDL. Cells prepared for indirect immunofluorescence with antibody against LDL and labeled with dil(3)-LDL have coincident antibody and dil(3)-LDL fluorescence distributions. HMG-CoA reductase is inhibited by dil(3)-LDL, indicating that the LDL must retain its cholesterol ester. Furthermore, we present electron microscopic and spectroscopic results and theoretical arguments which indicate that the dil(3) probably resides in the LDL-phospholipid shell. In addition, the dil(3)-LDL fluorescence distribution on normal (GM-3348), LDL receptor-negative (GM-2000), and LDL receptor internalization-defective human fibroblasts (GM-2408A) are examined and compared to each other and to previous immunofluorescence results (17, 25). By using these three cell lines as references, we demonstrate that dil(3)-LDL can be prevented from binding to its receptor by an excess of unlabeled LDL and displaced from its receptor by heparin sulfate (30).

MATERIALS AND METHODS

LDL Purification

LDL was purified by density gradient centrifugation (9, 20, 30, 31). Fresh plasma (<48 h old) was obtained from the Red Cross in Syracuse, N. Y.

Reconstitution of LDL with dil(3)

Three procedures to incorporate the lipid analog dil(3) into LDL are described. The first, method I, is essentially that of Kreiger et al. (20, 21). The second, method II, is the procedure of choice. With this procedure the LDL retains the cholesterol esters. The third procedure, method III, was used to test whether dil(3) could partition into the LDL-phospholipid shell from an aqueous environment.

Method I

LDL was prepared for reconstitution by the procedure of Kreiger et al. (20, 21). 2 mg of LDL in solution were combined with 25 mg of insoluble potato starch (Sigma Chemical Co., St. Louis, Mo.) in a Siliclad-treated 13- × 100-mm test tube (Clay Adams, Div. of Becton, Dickinson & Co., Parsippany, N. J.). The tubes were vortexed, rapidly frozen in liquid nitrogen, and lyophilized in a vacuum of 1–5 μm. The lyophilized material was stored in a desiccator over P₂O₅ at 4°C until use.

The lyophilized LDL was extracted twice for 1 and 0.5 h, respectively, by adding 5 ml of –20°C heptane to each tube, vortexing every 10 min, and incubating at –20°C. The tubes were centrifuged after each incubation at 2,000 rpm in a Beckman type 40 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 4°C for 10 min, and the supernate was removed. After the second centrifugation, the pellet was incubated at –20°C for 1.5–2.5 h with 4.5 mg of dil(3), dried from a freshly prepared solution of dil(3) in methanol, in 0.4 ml of –20°C heptane. The mixture was evaporated to dryness over an ice bath with a stream of dry nitrogen. 1 ml of 10 mM Tricine, 0.01% azide at pH 8.2 was added and each tube was incubated for 41 h at 4°C with occasional shaking to resuspend material. The tubes were then centrifuged 10–15 min at 2,000 rpm at 4°C, and the supernate was removed. The supernate had the red color of dil(3) and contained the reconstituted LDL. It was centrifuged twice in clean tubes for 20 min each at 12,000 rpm in a Beckman type 40 rotor to remove traces of suspended matter. The dil(3)-LDL was stored at 4°C in plastic microfuge tubes until use. Based on results of a Lowry protein assay (32), the yield of reconstituted LDL averaged 8–12%.

Method II—Addition of NaCl

Two mg of lyophilized LDL were combined with 4.5 mg of dil(3) in 0.4–0.6 ml of –20°C heptane, incubated, evaporated to dryness, and extracted as previously described but with a buffer consisting of 0.12 N NaCl, 10 mM Tricine, and 0.01% azide, pH 8.2. After centrifugation to remove the starch, the yield of LDL from a single extraction based on a Lowry assay was 40–47% of the starting protein.

Method III—Dil(3) Labeling of LDL from Solution

15 μl of a 10 mg/ml solution of dil(3) in methanol was added for 1 h to a 0.5-ml solution containing 2 mg of LDL and 25 mg of insoluble potato starch. The mixture was rapidly frozen in liquid nitrogen, lyophilized, and purified as described above for dil(3)-LDL.

Growth and Treatment of Cells

All procedures involving cells were carried out under sterile conditions in a laminar flow hood (Baker Co., Sanford, Maine). Three human fibroblastic cell lines, the mutants GM-2000 and GM-2408A and the normal GM-3348, were obtained from the Human Genetic Mutant Cell Repository (Camden, N. J.). Cells used for experiments were grown in stock 75 cm² flasks (Corning Medical, Corning Glass Works, Corning, N. Y.) at 37°C in a 5% CO₂ atmosphere incubator (model 8241) (National Appliance Co., Portland, Oreg.) in a solution of Ham's F-12 supplemented with 10% Fetal Calf Serum (FCS). Cells were plated onto #1 22 × 31 cm² glass cover slips, 4/100 cm² petri dish, at densities of 10⁵ cell per dish. To remove LDL from the growth medium to increase the number of receptors per cell (27), the medium was removed after 24–48 h, the cells were washed once with Medium 199 (Grand Island Biological Co., Grand Island, N. Y. [GIBCO]), and 7–8 ml of delipidated LDL (27) was added per dish. Cells were allowed to incubate for an additional 40–70 h in this environment before being used for experimentation. Young cultures, in 2-ml sealed glass ampules at cell densities of 5 × 10⁵ cells/ml, were stored frozen in a dewar (Minnesota Valley Engineering, New Prague, Minn.) of liquid nitrogen. Cells were first placed in a solution of 10% glycerol, 20% FCS, in Ham's F-12. Aliquots of 1 ml were sealed in glass ampules, wrapped in cotton, and slowly frozen over dry ice over a period of 3–5 h. The vials were then stored in liquid nitrogen until use. Fresh cultures were restarted by quickly thawing a vial in a 37°C water bath, transferring the contents to a 50-ml plastic centrifuge tube to which 20 ml of fresh growth medium was added, and spinning the contents at 250 × g for 10 min. The supernate was removed and the pellet of cells was resuspended in 8 ml of growth medium and transferred to a Corning T-25 flask.

Cell Labeling 4°C

All solutions were kept at 0°–4°C. Cells were washed three times in phosphate-buffered saline (PBS) and once more in a buffer consisting of Medium 199

(GIBCO) supplemented with 10 mM HEPES at pH 7.3 (buffer A). Stock solutions of diI(3)-LDL were diluted to 6–12 $\mu\text{g}/\text{ml}$ of LDL in buffer A. Cells on glass cover slips were incubated with diI(3)-LDL at this concentration for 1–2 h at 4°C. They were then washed three times in PBS and incubated for 10 min in Hank's balanced salt solution (HBSS; GIBCO) supplemented with 2 mg/ml albumin (Sigma), 2 mM calcium, and 10 mM Tris at pH 7.3. Cells were then washed one more time in buffer A.

37°C Cell Labeling

Cells were treated as at 4°C, except for the following modifications. (a) Cells were first labeled at 4°C, warmed to 37°C for the indicated period of time, rapidly chilled by applying cold PBS, and then processed further at 4°C. (b) Cells were washed at 4°C, labeled for the indicated period of time at 37°C, rapidly chilled, and then processed further at 4°C.

Cell Fixation

Cells on glass cover slips were fixed in a 4% solution of formaldehyde in PBS for 10 min at 22°C, washed three times, and mounted for viewing in either buffer A or PBS.

Antibody Labeling

Fluorescein-conjugated rabbit anti-LDL (Ra-LDL) and fluorescein-conjugated goat anti-rabbit antibody (GAR) were obtained from N. L. Cappel Laboratories, Cochranville, Pa. GAR antibody was preabsorbed on seven cover slips of cells for 30 min per cover slip to remove antibodies with strong affinity for cell surfaces that would otherwise lead to nonspecific labeling. DiI(3)-LDL or LDL-labeled cells were treated at 4°C for 40 min with a 0.033 mg/ml solution of Ra-LDL, washed three times in PBS, incubated for 40 min at 4°C with 0.2 mg/ml GAR, washed three times in PBS, one time in HBSS containing 10 mM Tris and 2 mg/ml albumin monomer (Miles Laboratories, Inc., Elkhart, Indiana) for 10 min, and then fixed in formaldehyde.

HMG-CoA Reductase Assay

The assay was performed in the laboratory of Drs. Michael Brown and Joseph Goldstein (University of Texas Health Science Center). Cells were grown in lipoprotein-deficient serum for 48 h and then incubated in minimum essential medium plus 10% human lipoprotein-deficient serum plus penicillin and streptomycin. The lipoproteins were added to a final concentration of 10 $\mu\text{g}/\text{ml}$ and the cells were incubated for 7 h. The cells were then harvested by scraping and dissolved in the nonionic detergent KYRO-EOB (Proctor and Gamble, Cincinnati, Ohio). HMG-CoA reductase activity was assayed as has been reported (27, 28).

Electron Microscopy

Samples of LDL and diI(3)-LDL were negatively stained in a 2% solution of sodium phosphotungstate, pH 7, and mounted and dried on carbon-coated grids.

DiI(3) Concentration in LDL

The concentration of diI(3) in LDL was measured by placing a known amount of diI(3)-LDL (by protein) in 3 ml of ethanol and comparing the absorption at 552 nm with a solution of 3.3 $\mu\text{g}/\text{ml}$ of diI(3) in ethanol. The ratio of dye to LDL was computed by assuming a mass ratio of 1,000 (33) for diI(3) and 600,000 (22) for the LDL protein.

Spectroscopy of DiI(3) in Solution

Equally concentrated samples of diI(3) in ethanol, octane, and water were made by diluting a 0.5 mg/ml ethanol stock solution of diI(3) 150 times. The concentration of diI(3) in a sample of diI(3)-LDL in water was calculated to equal the diI(3) concentration of the other samples. Absorption spectra were measured on a Cary dual beam spectrophotometer (Varian, Florham Park, N. J.) and were corrected for the characteristic absorption of the sample cuvettes.

Photography

Cells were photographed on a Nikon Optiphot microscope with fluorescence optics. DiI(3) fluorescence was excited at 520–550 nm and detected through a 580-nm barrier filter. Fluorescein fluorescence was excited at 450–480 nm and

photographed through a 515-nm barrier filter to block the exciting light, and with a $5,259 \pm 220 \text{ \AA}$ interference filter (Ditric Optics, Inc., Hudson, Mass.) placed directly in front of the camera.

RESULTS

DiI(3)-LDL antigenicity and binding specificity were checked by indirect immunofluorescence (Fig. 1). Cells were labeled at 4°C with diI(3)-LDL (Fig. 1a) and then prepared for indirect immunofluorescence with a fluorescein-labeled goat anti-rabbit antibody against rabbit anti-LDL. The second antibody was preabsorbed against seven cover slips of cells to prevent non-specific antibody binding. Even though the fluorescein fluorescence that appears green can be distinguished from the orange-red fluorescence of diI(3), an interference filter centered at 5,259 \AA with a 440 \AA bandwidth and 73% peak transmission was placed directly before the camera lens and above the barrier filter during the photography of the fluorescein fluorescence. DiI(3) excited at 450–480 nm still fluoresces slightly (orange), but the intensity is much less than that of the fluorescein fluorescence intensity. The interference filter completely blocks all diI(3) fluorescence excited at either 450–480 or 520–550 nm. Unfortunately, it also reduced the fluorescence intensity of the fluorescein fluorescence two- to threefold. Comparison of the fluorescence from diI(3)-LDL excited at 520–550 nm (Fig. 1a) to that of the fluorescein-labeled antibody fluorescence excited at 450–480 nm (Fig. 1b) shows an identical distribution. This is easily seen by beginning at the arrow which points to two fluorescent spots on Fig. 1a and following the fluorescence pattern along the length of the cell. As a test of antibody specificity, cells in which either the diI(3)-LDL or first antibody incubation was omitted produced no staining when exposed to the fluorescein goat anti-rabbit IgG (results not shown). Cells labeled with native LDL and then processed for indirect immunofluorescence had fluorescence distributions similar to that of diI(3)-LDL, except that they were more intense. We attribute this slight loss of intensity to quenching of fluorescein fluorescence by diI(3) incorporated in the LDL.

In Fig. 1c are Ouchterlony immunodiffusion results which confirm that diI(3)-LDL possess antigenicity similar to that of LDL. Wells 1–5 contain rabbit anti-LDL (2 mg/ml), LDL (5 mg/ml), goat anti-rabbit IgG (2 mg/ml), goat anti-rabbit IgG (2 mg/ml), and diI(3)-LDL (1.2 mg/ml), respectively. Both LDL and diI(3)-LDL form precipitant bands with rabbit anti-LDL, but not with goat anti-rabbit IgG. Goat anti-rabbit antibody in wells 3 and 4 precipitated in a heavy band with rabbit antibody from well 1. (The halo about wells 3 and 4 is due to reflections from the dish.) When the precipitant band in front of well 5 was examined under the fluorescence microscope with 520–550-nm excitation, the characteristic orange-red fluorescence of diI(3) was observed to coincide with the precipitate. These results indicate that diI(3)-LDL possesses antigenicity similar to that of LDL, and that the diI(3) fluorescence originates from the locations where LDL is bound.

In Fig. 2 are micrographs of negatively stained native LDL and diI(3)-LDL prepared by the three procedures described in Materials and Methods. Fig. 2a is a micrograph of negatively stained LDL. The particles are similar in size and appearance to LDL described in other reports (9, 24, 26). Fig. 2b shows diI(3)-LDL made by method II. Its structure is similar to that of native LDL in Fig. 2a, indicating retention of cholesterol ester (26). An occasional collapsed particle of LDL can be seen in Fig. 2b. To test whether the diI(3) would incorporate into

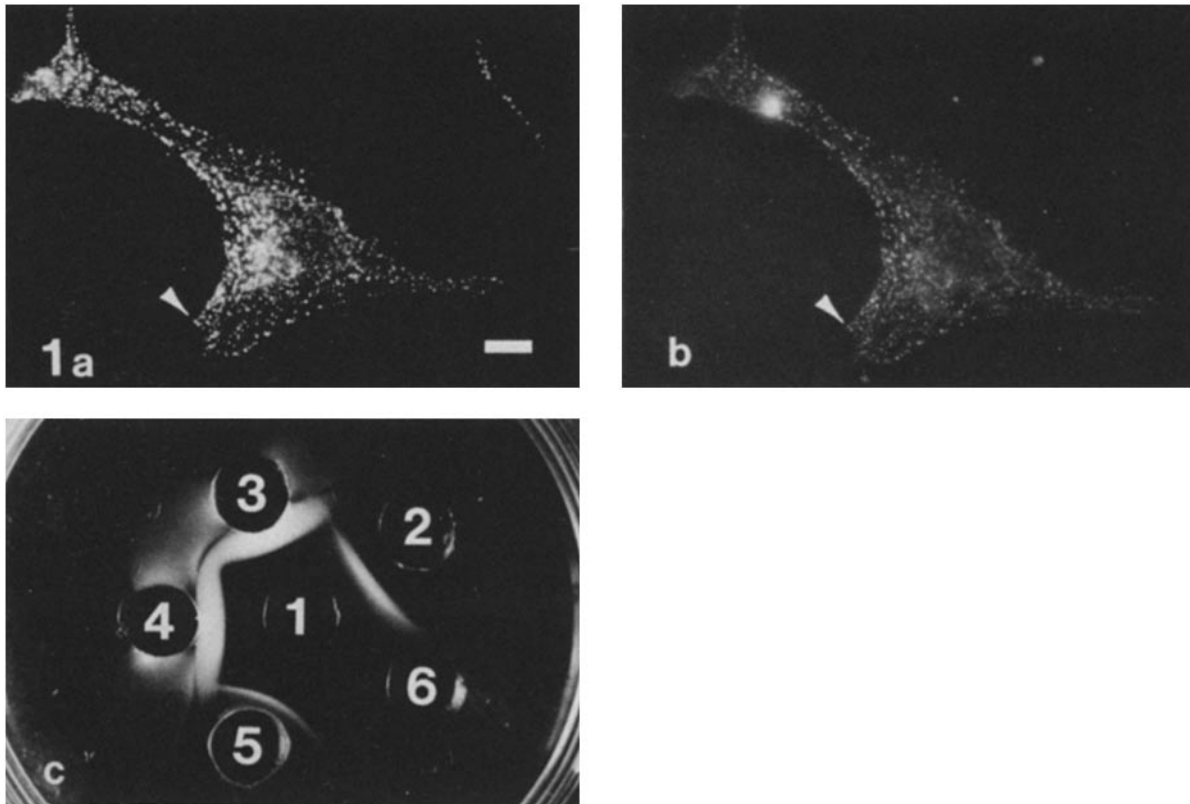


FIGURE 1 (a) GM-3348 human fibroblasts labeled with $12 \mu\text{g/ml}$ of $\text{dil}(3)\text{-LDL}$ at 4°C for 2 h and then processed at 4°C for indirect immunofluorescence with fluorescein-conjugated antibody against anti-LDL antibody. This micrograph shows the distribution of $\text{dil}(3)\text{-LDL}$ in $\text{dil}(3)$ fluorescence excited between 520 and 550 nm. (b) The same cell as in a but the fluorescence distribution of particles is now due to fluorescein excitation between 450 and 480 nm. This micrograph was taken through an interference filter centered at $5,259 \text{ \AA}$ that eliminated all $\text{dil}(3)$ fluorescence regardless of the excitation wavelength. By beginning at the arrows, a one-to-one correspondence can be observed between the fluorescence distributions of a and b. (c) In this Ouchterlony immunodiffusion plate, wells 1–5 contain, respectively, 2 mg/ml rabbit anti-LDL, 5 mg/ml LDL, 2 mg/ml GAR, 2 mg/ml GAR, and 1.2 mg/ml $\text{dil}(3)\text{-LDL}$. Both LDL and $\text{dil}(3)\text{-LDL}$ precipitated with rabbit anti-LDL, but not with GAR. The precipitan band in front of well 5 also had the characteristic fluorescence of $\text{dil}(3)$ when excited at 520–550 nm. Bar, $8.5 \mu\text{m}$.

the LDL-phospholipid matrix we added $\text{dil}(3)$ in methanol to a solution of LDL. The methanol injection was required due to the insolubility of $\text{dil}(3)$ in water (33). The resulting $\text{dil}(3)\text{-LDL}$ is shown in Fig. 2c. As is evident, the normal LDL structure has remained. This derivative bound to fibroblasts. Fig. 2d shows $\text{dil}(3)\text{-LDL}$ made by method I, in which the LDL undergoes heptane extraction (shown at smaller magnification than Fig. 2a–c). All the particles are collapsed, indicating that the cholesterol esters have been removed (26).

Spectroscopic measurements indicated that ~ 45 molecules of $\text{dil}(3)$ were incorporated into each molecule of LDL. This corresponds to 6–7% of the total phospholipid per LDL (24), and would place one $\text{dil}(3)$ molecule every 50 \AA in the LDL shell surface if the molecules were homogeneously distributed.

In Fig. 3 are absorption spectra of equal concentrations of $\text{dil}(3)$ in water, ethanol, octane, and as $\text{dil}(3)\text{-LDL}$ in water. For ethanol solutions of $\text{dil}(3)$, this concentration falls in the linear range of an optical density (552 nm) vs. concentration plot (results not shown). The relative absorption peak heights at 552 nm, $A(552)$, reflect the changes in extinction coefficients for $\text{dil}(3)$ due to changes of solvent polarizability. The ratio of peak intensities, $A(552)/A(514)$, is inversely related to the dimerization state (33) of the $\text{dil}(3)$. We conclude from these ratios that the $\text{dil}(3)$ in LDL is mostly in monomer form. The peak heights at 552 nm for $\text{dil}(3)$ associated with the LDL

show that it is not completely submerged in the hydrophobic interior of the LDL, since the peak intensity at 552 nm is in between the values for $\text{dil}(3)$ in water and the reduced value at 552 in octane. These spectra are consistent with a placement of $\text{dil}(3)$ in LDL, like a phospholipid with the polar head group exposed to water and the hydrophobic carbon chain in the interior.

The inhibitory effects of $\text{dil}(3)\text{-LDL}$ and normal LDL on the activity of HMG-CoA reductase were compared. Method II $\text{dil}(3)\text{-LDL}$ is almost as effective as unextracted LDL in inhibiting this enzyme. It inhibited HMG-CoA reductase by $>80\%$ in human fibroblasts when applied at a concentration of $10 \mu\text{g/ml}$. Some of the activity difference can probably be accounted for by a loss of cholesterol ester from LDL during the $\text{dil}(3)$ incorporation procedure, rather than any loss of $\text{dil}(3)\text{-LDL}$ binding affinity.

Fig. 4 presents fluorescence and phase-contrast micrographs of normal and mutant human fibroblasts labeled with $\text{dil}(3)\text{-LDL}$. There were no differences in cell staining by $\text{dil}(3)\text{-LDL}$. There were no differences in cell staining by $\text{dil}(3)\text{-LDL}$ made by either method I or II. Therefore the surface binding distribution and internalization properties of $\text{dil}(3)\text{-LDL}$ appear indifferent to the loss of cholesterol ester produced by method I as expected for LDL (26). Fig. 4a shows labeled cells from normal GM-3348 human fibroblasts. Most of the fluorescent

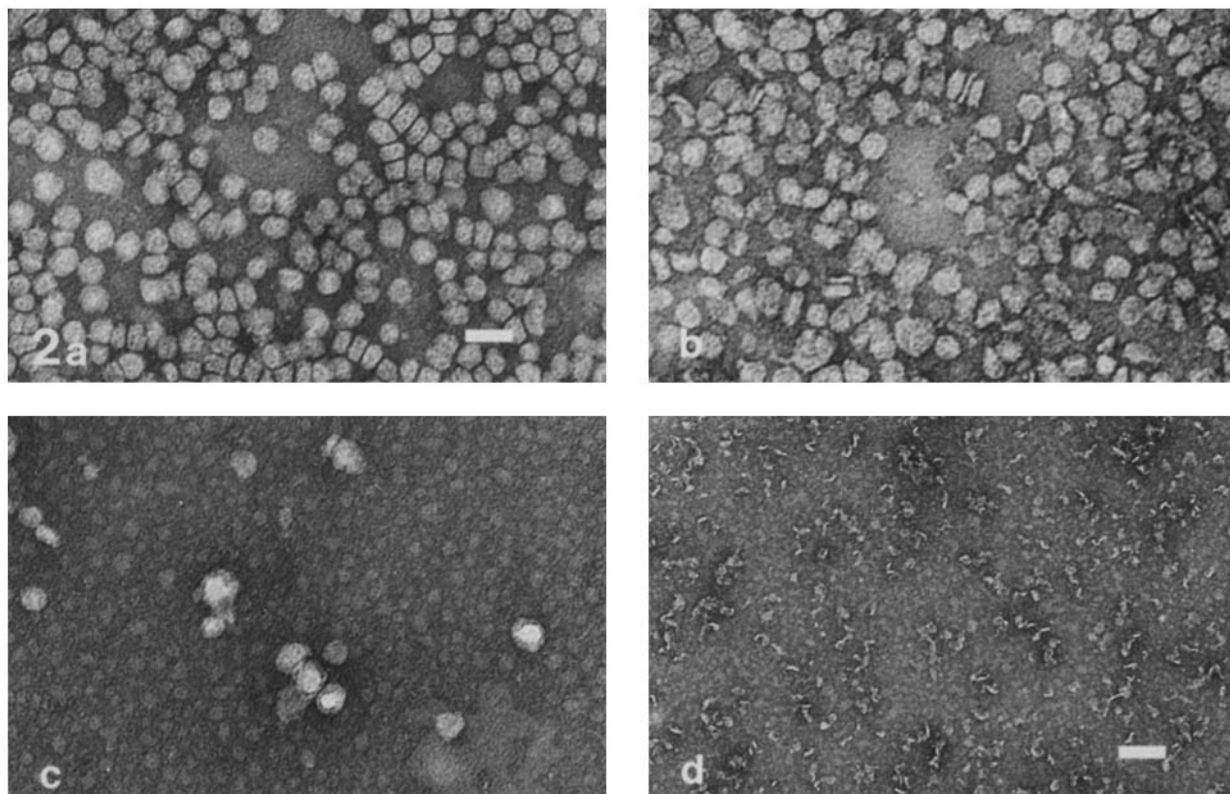


FIGURE 2 Electron micrographs of negatively stained samples of LDL (a), diI(3)-LDL prepared by method II (b), diI(3)-LDL prepared by method III (larger particles against background) (c), and diI(3)-LDL prepared by method I (d). The collapse of the structure of LDL seen in (d) is due to removal of cholesterol ester from the LDL shell. a-c: bar, 368 Å; d: bar, 140 Å.

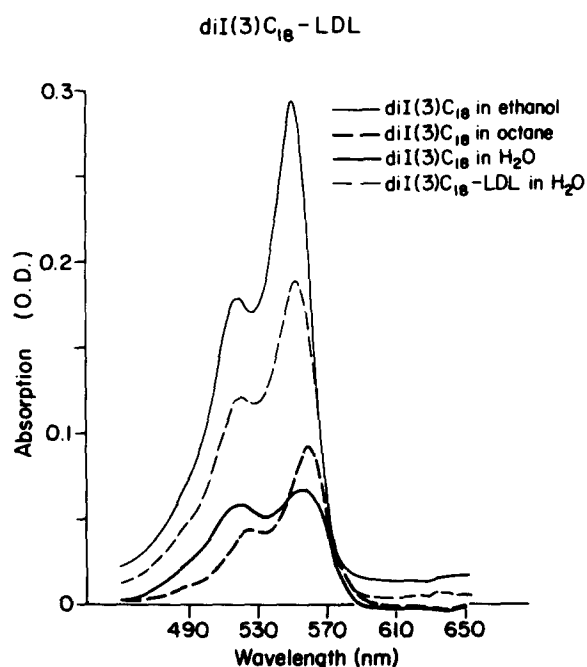


FIGURE 3 Spectra of equal concentrations of diI(3) in water, octane, ethanol, and as diI(3)-LDL in water. These spectra are consistent with a phospholipid-type orientation of diI(3) in the shell of LDL (see text).

dots are expected to correspond to three to five LDL receptors located in coated areas of membrane (25). The fluorescence was extremely bright and has a distribution similar to that seen on normal fibroblasts with 3-pyrenemethyl-23,24-dinor-5 cho-

len-22-oate-3 betayl oleate (PMCA oleate)-LDL or indirect immunofluorescence labeling of bound LDL (25). Normal GM-3348 fibroblasts treated in our laboratory with PMCA oleate reconstituted LDL (a gift of Monty Kreiger) produced a fluorescence distribution similar to that in Fig. 4a. The cells illustrated in Fig. 4c and d, and e and f, were treated with diI(3)-LDL and photographed under identical conditions. The cells in Fig. 4c and d are GM-2408A mutant fibroblasts which have a homogeneous distribution of surface LDL receptors. According to Anderson et al. (7), these receptors are not preferentially located in coated pits but are distributed as individual or groups of two or three receptors (25). Thus the fluorescent dots are expected to correspond to from 1 to 3 LDL molecules. The receptor-negative mutants, GM-2000, do not bind LDL. This is clearly demonstrated in Fig. 4e, which was exposed and printed under conditions identical to those in Fig. 4c.

Mutant GM-2408A and normal GM-3348 cells were labeled with diI(3)-LDL at 4°C. Comparison of the fluorescence distribution between them (not shown) confirms observations (17, 25) that LDL on normal human fibroblasts often appears in linear arrays, while LDL on GM-2408A in most cases seems to be homogeneously dispersed. We have observed occasional mutant GM-2408A cells which have receptors arrayed in a linear pattern.

Fig. 5a and b show GM-2408A cells that were photographed and printed under identical conditions, but the labeling differed in that the cell in Fig. 5b was simultaneously exposed to a 100-fold excess of unlabeled LDL during diI(3)-LDL labeling at 4°C. To determine that the displacement of diI(3)-LDL in Fig. 5b was not simply due to excess protein in the labeling medium, cells were labeled with our standard conditions in the

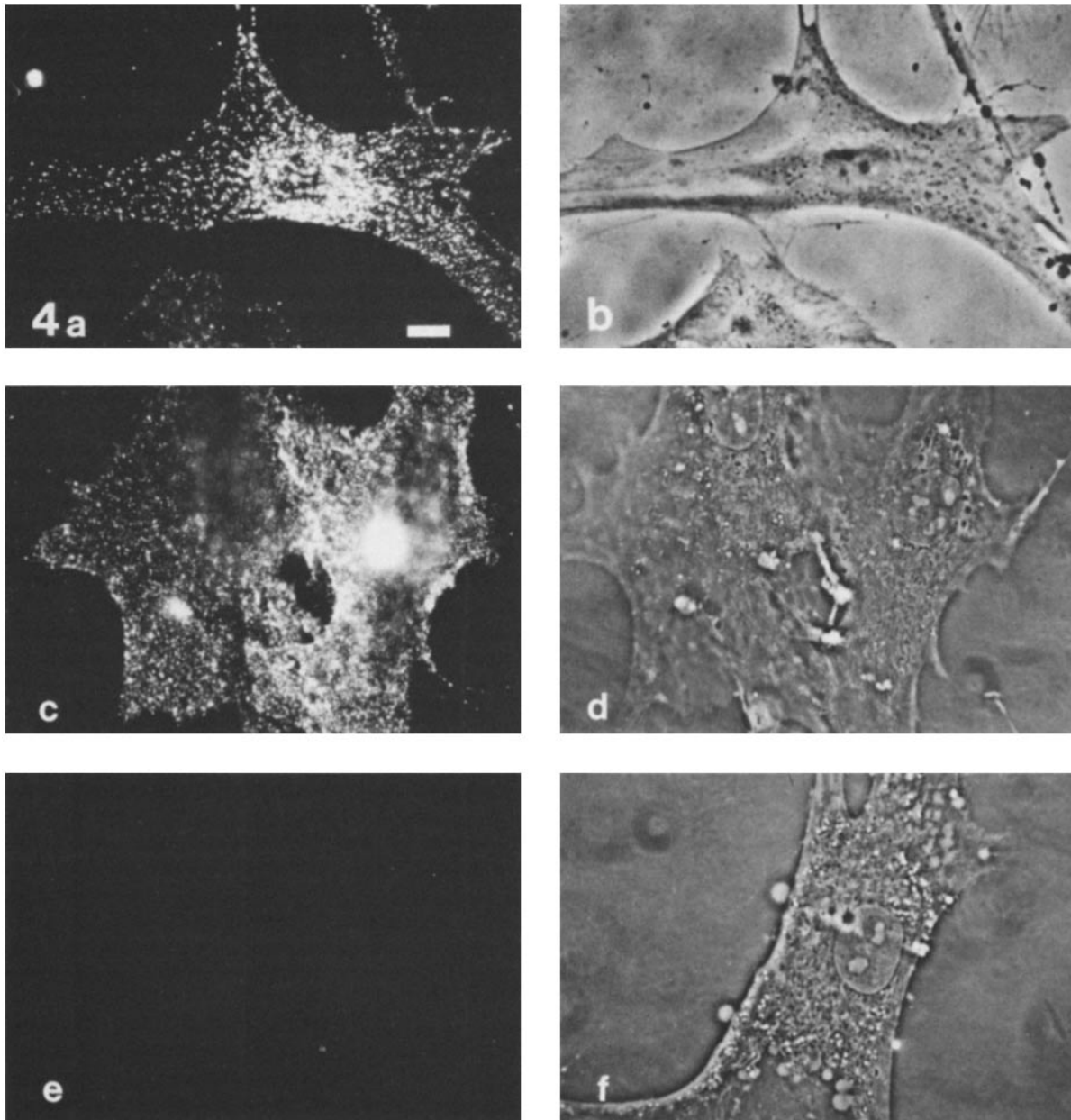


FIGURE 4 Alternate fluorescence and phase-contrast micrographs of diI(3)-LDL fluorescence on normal human fibroblasts (a and b); GM-2408A LDL-receptor internalization-defective human fibroblasts (c and d); and GM-2000 LDL-receptor-negative human fibroblasts (e and f). Bar, 8.5 μ m.

presence of 2 mg/ml albumin. They showed fluorescence patterns similar to those in Fig. 5a.

Quantitative photocount measurements of the absolute integrated fluorescent intensity were made on GM-2408A cells treated as in Fig. 5a and b. Results are presented in Table I. After correction for cellular autofluorescence, which includes unlabeled native LDL fluorescence, the total nonspecific fluorescence intensity of diI(3)-LDL treated cells is only 0.5–3% of the total fluorescence. Thus, within the uncertainty of the measurement, an 80-fold excess of native LDL completely prevents diI(3)-LDL binding.

To insure that the diI(3)-LDL-labeling patterns were not somehow changed by receptor redistribution during 4°C incubations, cells were first fixed with formaldehyde and then labeled. Both GM-2408A and GM-3348 displayed LDL fluo-

rescence patterns identical to those already shown for live cells. Cells labeled at 4°C, kept at 4°C, and then examined on a 4°C temperature-controlled microscope stage also produced labeling patterns identical to those seen in Figs. 1–3.

Fig. 6 shows cells that were exposed to temperatures of 37°C during or after labeling with diI(3)-LDL. Each respective pair (a–b, c–d, e–f) was photographed and printed under identical conditions. Fig. 6a shows GM-2408A cells that were labeled for 15 min with diI(3)-LDL, whereas the cell in Fig. 6b was simultaneously treated with a 100-fold excess of unlabeled LDL in addition to diI(3)-LDL. The slight fluorescence seen in Fig. 6b is probably due to diI(3)-LDL internalized nonspecifically. The fluorescence is visible as a result of the extreme brightness of single diI(3)-LDL particles. The pair in 6c and d represents GM-2408A cells that were labeled for 15 min at

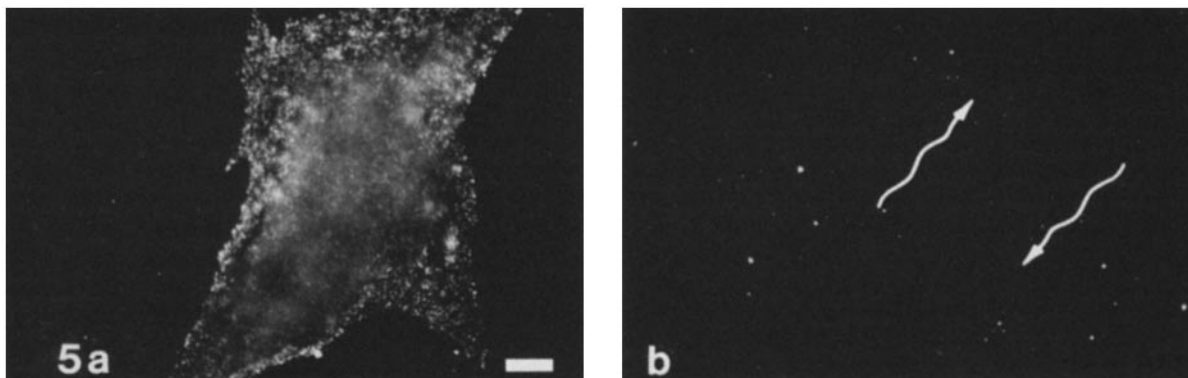


FIGURE 5 (a) GM-2408A human fibroblasts labeled at 4°C for 2 h with 12 $\mu\text{g}/\text{ml}$ of diI(3)-LDL. (b) GM-2408A labeled as in a with 12 $\mu\text{g}/\text{ml}$ of diI(3)-LDL plus a 100-fold excess of unlabeled LDL. These micrographs were exposed and printed under identical conditions. The arrows in b indicate the border of the cell. LDL completely prevented the binding of diI(3)-LDL in b, whereas diI(3)-LDL binding was not prevented by 2 mg/ml albumin. The surface of the center of the cell in a is not quite in focus due to the limited depth of field of the objective lens. This results in a defocused fluorescence image from this area in contrast to the cell periphery. Bar, 8.5 μm .

TABLE I
Specificity of DiI(3)-LDL Binding to Fibroblasts

Samples	diI(3)-	LDL	Photocounts
	$\mu\text{g}/\text{ml}$	mg/ml	
GM-2408A	12	—	90,800 \pm 33,400
GM-2408A	12	1	7,640 \pm 1,657
GM-2408A	—	1	7,200 \pm 2,570
GM-2408A	—	—	5,350 \pm 1,790

Cells were washed and labeled at 4°C with the indicated amounts of LDL and diI(3)-LDL as described in Materials and Methods. All photocount results are the average of 10 measurements with each measurement performed on a different cell. Intensities were measured by illuminating an area which remained constant on all samples with the 514-nm line of an argon-ion laser directed through a $\times 100$ phase-objective of a Zeiss Universal microscope. Fluorescence intensity was monitored through a Schott OG530 barrier filter by an RCA C31-034 photomultiplier (34).

37°C with and without 5 mg/ml of heparin sulfate. The heparin completely displaced the LDL from its receptor. Heparin also displaced and prevented the binding of LDL at 4°C when incubated simultaneously with or subsequent to diI(3)-LDL incubation (results not shown). The pair Fig. 6e and f show GM-3348 and GM-2000 cells that were first labeled with diI(3)-LDL at 4°C, washed, and then incubated at 37°C for 15 min. The normal GM-3348 fibroblasts in Fig. 6e rapidly internalized the diI(3)-LDL. The fluorescence probably originates from lysosomes (7) that are becoming distributed about the nucleus. The GM-2000 cells of Fig. 6f are not fluorescent due to the lack of LDL binding at 4°C.

The fluorescence distribution of diI(3)-LDL on Fig. 6e is much more heterogeneous than that of either Fig. 6a or c. Labeling has disappeared from peripheral areas of the cell in Fig. 6e, whereas in the GM-2408A mutants the peripheral and general surface labelings are homogeneous enough to delineate the cell. It is evident from the micrographs that the sizes of the fluorescent clusters in Fig. 6a and c are smaller than those of Fig. 6e. The difference between mutants and normals is also true of GM-2408A first labeled at 4°C and then incubated at 37°C for 15–20 min. Exposure to 37°C for 15–20 min is enough to produce some fluorescent clusters in the GM-2408A mutants that appear to be internalized as seen by exclusion from the nucleus in some cases and focus below the cell membrane. We

estimate that the fluorescent clusters contain in general <10% of the homogeneously bound diI(3)-LDL remaining with the cell. In contrast the normal cells internalize all peripherally bound diI(3)-LDL.

DISCUSSION

The diI(3)-LDL developed and used here has the antigenic, enzyme inhibition, binding, and internalization properties expected of normal LDL. Antibody labeling of diI(3)-LDL indicates that it contains antigenicity similar to that of normal LDL. The indirect immunofluorescence experiment shows directly that the diI(3) remains associated with the LDL once the complex binds. This association is also indicated by Ouchterlony immunodiffusion and the large concentration of diI(3) in solution with LDL (87 $\mu\text{g}/\text{ml}$), since diI(3) is water insoluble (32).

Three methods are described to incorporate diI(3) into LDL. Method II evolved from method I and was developed for the following reasons. We believed that because the chromophore of diI(3) is charged (Fig. 7) it is probably not soluble in the nonpolar interior of LDL. In addition, Kreiger et al. have shown that esters of long-chain saturated fatty acids cannot be incorporated into the core of LDL. Therefore, it is not necessary to remove the cholesterol esters from the LDL, because they are not being replaced by the diI(3), and this lack of incorporation and failure to replace the cholesterol ester probably accounts for the collapsed appearance of LDL in Fig. 2d. We believed that the yield of diI(3)-LDL from starch in method I could be improved by salting in the diI(3)-LDL. High salt extractions (26) had previously been used to remove starch-bound LDL with 60% recovery. By extracting diI(3)-LDL with 0.12 N NaCl from starch, our yield of LDL protein increased six- to eightfold. The observation that diI(3) would incorporate into both native and extracted LDL (Fig. 2b and d) along with our belief that it was too charged to partition into the cholesterol ester fraction of LDL motivated method III. If diI(3) could directly incorporate into LDL from aqueous solution, it must first partition into the phospholipid layer. The results including incorporation by method III, comparison of the absorption spectra of diI(3)-LDL with diI(3) in solutions of differing polarity, its known charge, and its saturated fatty-acid carbon chains indicate diI(3) partitions into the LDL phospholipid shell.

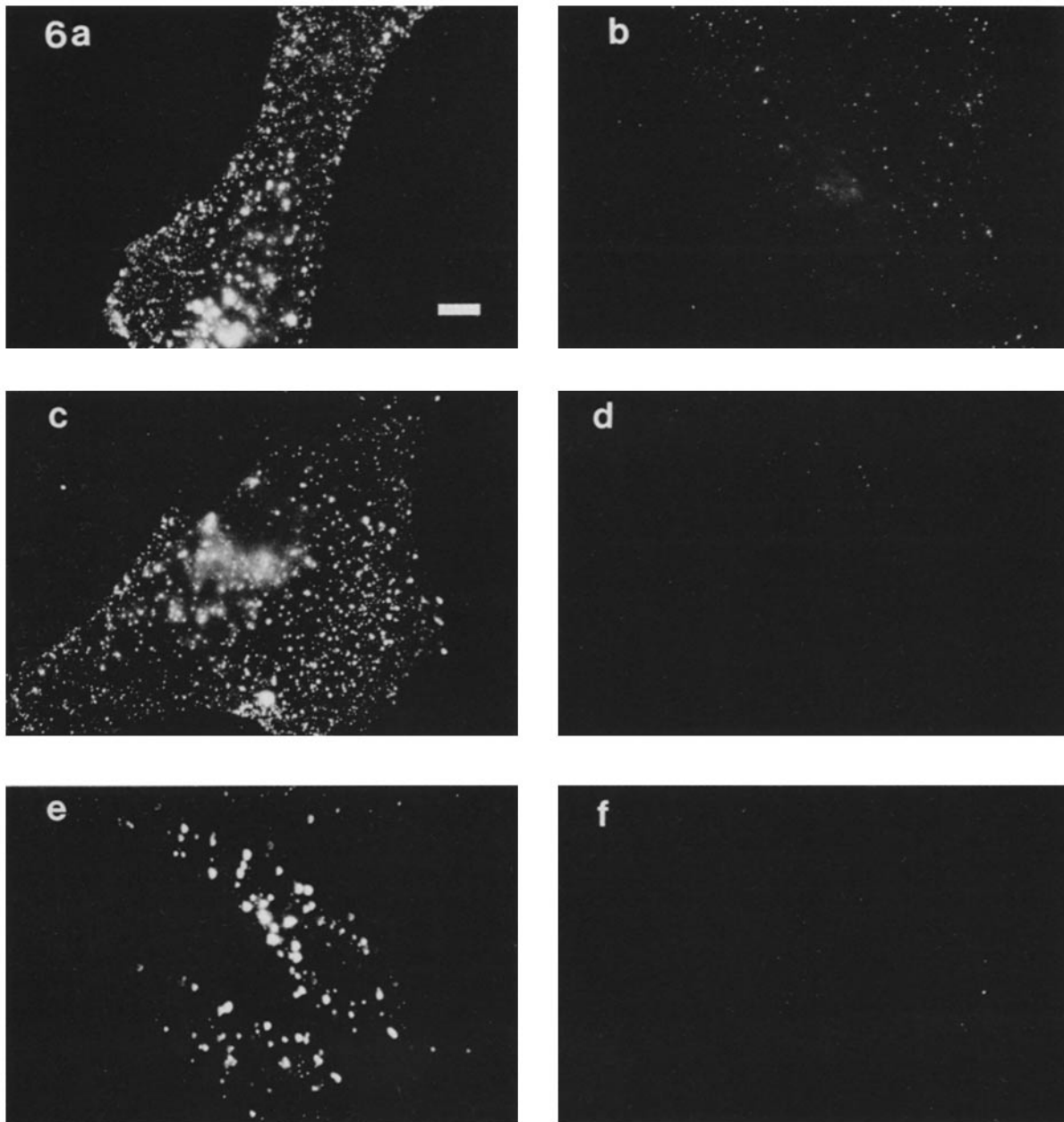


FIGURE 6 Each pair of micrographs (a and b), (c and d), and (e and f) was exposed and printed under identical conditions. (a) GM-2408A fibroblasts labeled with 12 $\mu\text{g}/\text{ml}$ of diI(3)-LDL for 15 min at 37°C. (b) Cell treated as in a with 12 $\mu\text{g}/\text{ml}$ of diI(3)-LDL plus a 100-fold excess of unlabeled LDL. (c) GM-2408A treated as in a. (d) GM-2408A treated as in c with 12 $\mu\text{g}/\text{ml}$ diI(3)-LDL plus 5 mg/ml heparin sulfate. (e) Normal GM-3348 fibroblast labeled with 12 micrograms/ml diI(3)-LDL for 2 h at 4°C, incubated at 37°C for 15 min, and fixed in formaldehyde. (f) LDL receptor-negative GM-2000 fibroblast treated as in e. Bar, 8.5 μm .

We have shown that the fluorescence distribution of diI(3)-LDL on the three cell lines GM-3348, GM-2408A, and GM-2000 agree with results obtained by Anderson et al. using other fluorescence techniques (17, 25). The most sensitive assay involves incubation of the LDL receptor-negative GM-2000 line. At 4°C, this cell line exhibits no diI(3) fluorescence after incubation with diI(3)-LDL, indicating that negligible nonspecific fluorescence occurs upon labeling. This contrasts with the intense punctate labeling of the normal GM-3348 fibroblasts and the mutant GM-2408A fibroblasts. Comparison of diI(3) fluorescence between these two lines reveals a qualitative difference in fluorescence at 4°C and a gross difference at 37°C. The GM-2408A mutants retain some capability for internaliz-

ing diI(3)-LDL at 37°C. In contrast to the normal cells, the percentage internalized appears to be much less, and the distribution in the cell is slightly different. We do not know whether this involves another endocytotic pathway without coated vesicles, whether the LDL receptor in this instance happens to be located in a coated area by chance. This secondary process is only evident due to the extreme brightness of a few molecules of diI(3)-LDL. The diI(3)-LDL-labeling results at 4°C also confirm results (17, 25) that LDL receptors are prepatched on the membrane of normal cells and in most instances homogeneously distributed on the mutant line GM-2408A. GM-2408A cells occasionally do occur which have linear arrays of receptors. The vast majority of LDL receptors

Chemical Structure of diI(3)C₁₈

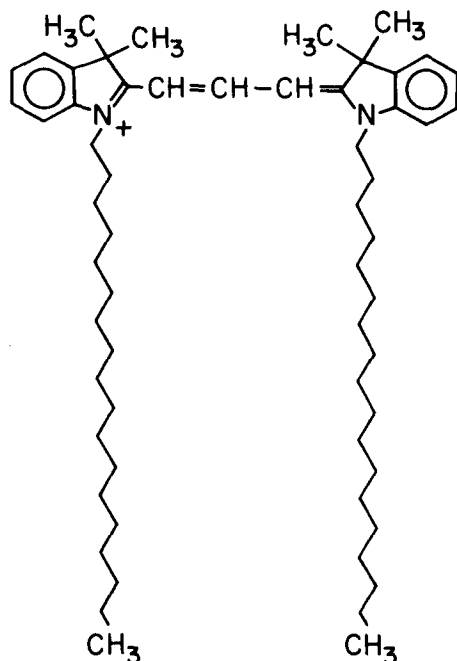


FIGURE 7 Structure of the lipid analogue diI(3)-LDL.

in this cell line are not associated with coated areas of membrane (25). It is not known whether the alignment in either case is due to a specific interaction with a cellular component.

Two other experiments confirmed the binding specificity of diI(3)-LDL. DiI(3)-LDL was prevented from binding by an excess of unlabeled LDL, and heparin sulfate (30) displaced or prevented diI(3)-LDL binding to the LDL receptor.

We have incorporated diI(3) into LDL for the purpose of studying receptor-ligand interactions and diffusion. The ~45 fluorophores incorporated per molecule allow receptor ligand complexes as small as 1-3 in number to be observed. The carbocyanine fluorophore is fairly photostable (33), and so multiple time-lapse observations can be made on the receptor complexes. Furthermore, the high concentration of fluorophore produces a large contrast between labeled and unlabeled areas of the cell surface. This permits experiments to be carried out in the presence of low receptor density.

When the receptor number is low, as for LDL, insulin, and other hormone systems (1, 2, 36, 37), the fluorophore number is especially critical. The number of fluorophores bound per unit area on the cell surface is a function of both receptor density and the stoichiometry between the ligand and conjugated dye. Rhodamine and fluorescein derivatives are the most common fluorescent conjugates. When attached to either LDL or the hormones mentioned above in equimolar ratios, fluorophore intensity is too low to permit fluorescence visualization of ligands in many geometries and cell types. This problem has been circumvented in one system by employing a cell line with abnormally high numbers of receptors (2). Other attempts to circumvent this numbers problem have relied on attaching more fluorescein or rhodamine dye groups to the ligand either directly or indirectly (35, 36, 37).

We believe that by incorporating a lipid-soluble dye or a cholesterol ester substitute into LDL, the arginine-and lysine-

associated (38) binding sites of LDL that is not being exposed to amino reactive dyes remain intact. The ratio of intensity between cells labeled with diI(3)-LDL and cells treated with diI(3)-LDL plus an 80-fold excess of LDL is between 50 and 200. This confirms that there is negligible nonspecific binding (39, 40) between the dye and cell-surface proteins other than LDL. Thus, it should be possible to covalently modify selected components of LDL, incorporate the diI(3), and determine the effects of the modification by observing changes in the fluorescence patterns compared to those of unmodified diI(3)-LDL. Experiments of this type appear possible because we have been able to observe individual LDL receptor complexes and or small clusters of 1-3 receptor complexes. This capability enables us to localize individual LDL-receptor complexes, and, in addition, to determine the statistical diffusion properties of the complex.

We have used the diI(3)-LDL to study receptor-ligand diffusion and internalization of GM-3348 and GM-2408A cells. Preliminary results indicate that the LDL-receptor complex is immobilized and that when freed from this linkage the LDL-receptor complex undergoes Brownian motion consistent with the Saffman-Delbruck (41, 42) diffusion theory for a free particle in two dimensions. This work is to be reported in a subsequent paper.

The complexity of the LDL molecule allows it to be labeled in a number of ways. The protein, cholesterol ester, and phospholipid portions can be labeled with 3 different probes to study both the internal environment of LDL and the path it follows after binding to its surface receptor. These fluorescence labeling techniques can also be applied to the other serum lipoproteins because they also contain lipid, cholesterol, and protein components.

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