Low density lipoproteins reconstituted with steroids containing the nitrobenzoxadiazole fluorophore

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Abstract A new cholesterol analog, N-(7-nitrobenz-2-oxa-1,3-diazole)-23,24-*dinor*-5-cholen-22-amine-3β-ol, with fluorescent properties similar to those of fluorescein, has been synthesized. The fluorescence lifetimes, quantum yields, and wavelength maxima of N-(7-nitrobenz-2-oxa-1,3-diazole)-23,24-*dinor*-5-cholen-22-amine-3β-ol and its linoleate ester were solvent-dependent. The cholesterol analog was a satisfactory substrate for lecithin:cholesterol acyltransferase. The fluorescent sterol, added in ethanol, gave half-maximal suppression of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in cultured human fibroblasts at 2.5 μM and was twice as effective as cholesterol. The fluorescent steryl ester, incorporated into low density lipoprotein, was used to demonstrate high affinity cellular uptake and degradation of the reconstituted lipoproteins, intracellular accumulation of the free sterol and simultaneous suppression of 3-hydroxy-3-methylglutaryl coenzyme A reductase in fibroblasts. Half-maximal suppression was achieved at 10 μg mL^{-1} of low density lipoproteins reconstituted with the fluorescent steryl ester, compared to the same degree of suppression produced by 2 μg mL^{-1} of native low density lipoproteins. The interaction of low density lipoproteins reconstituted with N-(7-nitrobenz-2-oxa-1,3-diazole)-23,24-*dinor*-5-cholen-22-amine-3β-ol linoleate with cells was readily visualized by fluorescence microscopy and quantified by fluorimetry. These analogs will facilitate the studies of lipoprotein-cell interactions and phospholipid vesicle-cell interactions, the selection of cell mutants defective in lipoprotein metabolism, and the assessment of the immediate environment of the steroids in cellular membranes.—**Craig, I. F., D. P. Via, W. W. Mantulin, H. J. Pownall, A. M. Gotto, Jr., and L. C. Smith.** Low density lipoproteins reconstituted with steroids containing the nitrobenzoxadiazole fluorophore. *J. Lipid Res.* 1981. **22:** 687–696.

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The cholesterol pool within the cell consists of endogenously synthesized cholesterol and cholesterol delivered to the cell by lipoproteins, especially by low density lipoproteins, LDL (1, 2). The observed relationship of LDL and atherosclerosis has stimulated great interest in the mechanisms by which lipoprotein cholesterol enters the cell (3). While the uptake and intracellular metabolism of LDL has been studied extensively with ^{125}I- and ferritin-labeled LDL (4), little information is available about the intracellular fate of the lipid components of LDL, due in part to the absence of suitable spectroscopic methods to visualize the intracellular distribution of these compounds. Accordingly, synthesis of a series of fluorescent sterols has been undertaken.

A good cholesterol analog must contain three main structural features: a 3β-hydroxyl group, a planar nucleus, and an intact chain at C-17 (5). A fluorescent analog containing a naphthyl group at C_{22}, N-(2-naphthyl)-23,24-*dinor*-5-cholen-22-amine-3β-ol, has been synthesized in this laboratory (6). This alteration of the side chain produced a sterol that exhibited appreciable activity as a substrate with enzymes that ordinarily utilize cholesterol as a substrate. The concentration-dependent fluorescence properties of a different cholesterol analog containing pyrene, 3-pyrenemethyl 23,24-*dinor*-5-cholen-22-oate-3β-ol, PMCA, have been used to demonstrate that the rate-limiting step in transfer of sterols between lipoproteins involves desorption of individual molecules into the

Abbreviations: LDL, low density lipoproteins; PMCA, 3-pyrenemethyl 23, 24-*dinor*-5-cholen-22-oate-3β-ol; NBD-C, N-(7-nitrobenz-2-oxa-1,3-diazole)-23,24-*dinor*-5-cholen-22-amine-3β-ol; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LPDS, lipoprotein deficient serum; NBD-CL, N-(7-nitrobenz-2-oxa-1,3-diazole)-23,24-*dinor*-5-cholen-22-amine-3β-yl linoleate; r-(NBD-CL)LDL, heptane-extracted LDL reconstituted with NBD-CL; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; ϕ_f, fluorescence quantum yield; LCAT, lecithin:cholesterol acyltransferase; apoA-I, the major apoprotein component of high density lipoproteins (1); DMPC, dimyristoylphosphatidylcholine; CO, cholesteryl oleate.

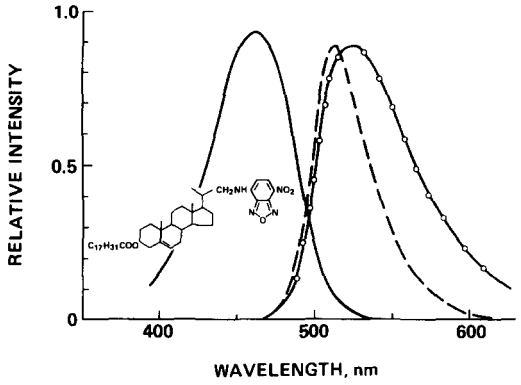

Fig. 1. Absorption (——), uncorrected (– – –), and corrected (○ —— ○) fluorescence spectra of NBD-CL in acetone.

aqueous solution (7). LDL reconstituted with PMCA oleate has been used to demonstrate uptake and intracellular distribution of the fluorescent probe (8, 9). It appears advantageous to have the ability to monitor simultaneously the intracellular fate of lipoproteins labeled with either pyrene-containing lipids or lipids that have fluorescence excitation and emission maxima in the visible region of the spectrum. Furthermore, with such compounds, commercially available microscopy systems that are designed for fluorescein-labeled proteins could be used.

In this study we report the synthesis and characterization of a new cholesterol analog, N-(7-nitrobenz-2-oxa-1,3-diazole)-23,24-*dinor*-5-cholen-22-amine-3β-ol, NBD-C, which contains the fluorescent N-(7-nitrobenz-2-oxa-1,3-diazole)amino fluorophore and therefore fluorescence properties similar to those of fluorescein (cf. **Fig. 1**). In addition, with LDL reconstituted with the linoleate ester of NBD-C, lipoprotein interaction with cells has been monitored directly by fluorescence microscopy. An abstract of this work has appeared (10).

MATERIALS AND METHODS

Cells

Human fibroblasts were established in our laboratory from skin biopsies of newborn foreskins and were maintained in Dulbecco's Modified Eagle's Medium containing 10% fetal calf serum supplemented with 1 mM L-glutamine, $NaHCO_3$, penicillin, and streptomycin as previously described (11). Cells were used between the fifth and ninth passages.

HMG-CoA reductase assay

Cells were grown to about 75% confluence in normal growth medium and incubated for an additional 24 hr in medium containing 5% LPDS (12). The desired quantities of lipoproteins or free sterol in ethanol were then added to duplicate dishes and incubated for 16 hr. After the cells were mechanically harvested, HMG-CoA reductase activities were determined by a modification (11) of the method of Brown, Dana, and Goldstein (12).

Lipoproteins

Human LDL (d 1.019–1.063 g mL^{-1}) and LPDS (d > 1.215 g mL^{-1}) from plasma of normolipemic donors or from bulk plasma obtained from The Methodist Hospital Blood Donor Center were isolated by sequential ultracentrifugation (13). Fluorescent LDL was prepared by a minor modification of the reconstitution method of Krieger et al. (14). LDL, 1.8 mg lyophilized with 25 mg starch, was extracted three times with 5 mL of heptane at −10°C. Following aspiration of the last heptane extract, 6 mg of steryl ester, either NBD-CL or a mixture of NBD-CL and cholesteryl oleate as indicated, was added in 200 μL of benzene. After 90 min at 4°C, benzene and any residual heptane were removed under a stream of N_2 in an ice salt bath. After about 45 min, no odor of the organic solvent in the dry powder could be detected. The r-LDL was solubilized in 10 mM Tricine, pH 8.2, at 4°C for 24 hr. Starch was removed from the solution by a low-speed centrifugation (500 *g*) followed by a 20-min centrifugation (6,000 *g*) in a Sorvall RC2 centrifuge. The preparation was finally filtered through a 0.8 μm Unipore filter (BioRad) to yield a clear yellow solution. The yields of LDL protein ranged from 48–52% in eight preparations. The amount of NBD-CL incorporated into the lipoprotein was determined by measurement of the absorption at 465 nm, using an observed molar absorption coefficient of 20,000 $M^{-1} \cdot cm^{-1}$. [^{125}I]iodo-LDL and r-[^{125}I]iodo-(NBD-CE)LDL were prepared with [^{125}I]Cl by the method of Shepard, Bedford, and Morgan (15), to give 200 cpm ng^{-1} protein. Protein content was determined by the method of Lowry et al. (16) modified by the inclusion of 0.1% sodium dodecyl sulfate (v/v) in all reagent solutions.

Uptake of fluorescent and [^{125}I]iodo-LDL

Fibroblasts were seeded into 60-mm culture dishes containing complete growth medium (11) at a density of 2×10^5 cells/plate. After 4 days, the medium was replaced with 3 mL 5% LPDS and incubated for a further 48 hr at 37°C. r-(NBD-CL)LDL were then added to a final concentration of 12 μg protein mL^{-1}. To show specificity, a 25-fold excess of unlabeled LDL, based on protein content, was added in some experiments. After incubation at 4°C or 37°C

for the times indicated in the figures, the plates were rapidly chilled, washed five times with buffer containing albumin, and subsequently incubated for 1 hr at 4°C with 1 mL of 10 mg mL^{-1} heparin in 10 mM Hepes, pH 7.4, containing 50 mM NaCl to release fluorescent LDL from the cell surface (17). The heparin washes were pooled for lyophilization. The remaining cell monolayers were scraped into PBS (18), and pelleted by centrifugation. After the addition of [4-^{14}C]cholesterol or [7(n)-^{3}H]cholesteryl oleate in 50 μL $CHCl_3$–CH_3OH 2:1 (v/v) to quantify extraction losses, NBD-CL was extracted with two aliquots of $CHCl_3$–CH_3OH 2:1 (v/v) from the cell pellets and lyophilized heparin washes. Extracts were dried under a stream of N_2 and redissolved in 1 mL of $CHCl_3$–CH_3OH 2:1 (v/v). An aliquot was removed for scintillation counting. The remaining sample was examined for fluorescence in a SLM Instruments Model 8000 photon counting spectrofluorimeter. With excitation at 465 nm, fluorescence emission was monitored at 525 nm with 1-sec accumulations. Fluorescence was quantified by comparison with standard curves of freshly prepared NBD-CL in $CHCl_3$–CH_3OH 2:1 (v/v). Background values obtained from parallel plates of cells incubated with non-fluorescent LDL were subtracted. Following the published procedures of Goldstein et al. (17), surface binding of [^{125}I]iodo-LDL was determined by counting [^{125}I]-iodo-LDL released by heparin. Internalized [^{125}I]-iodo-LDL was quantified by counting the radioactivity of the cell monolayers after dissolution in 1N NaOH. Degradation of [^{125}I]iodo-LDL was determined as soluble radioactivity in the culture medium remaining in solution after protein precipitation by trichloroacetic acid and chloroform extraction to remove minor amounts of ^{125}I associated with lipid (17).

Fluorescence microscopy

Fibroblasts were grown on sterile 18-mm^2 glass coverslips for incubation with either LDL or r-(NBD-CL)LDL. After the appropriate washings, the cells were fixed for 30 min at room temperature with 3% formaldehyde in PBS. Surface-bound LDL or r-(NBD-CL)LDL were visualized with affinity-purified rabbit anti-LDL IgG and FITC-conjugated goat anti-rabbit IgG. Internalized LDL was visualized by antibodies after treatment of cells with 0.05% Triton X-100 (19), while internalized r-(NBD-CL)LDL was visualized directly. All coverslips were mounted in a drop of 90% glycerol–10% PBS (v/v), pH 8.5, and examined with a Zeiss Photomicroscope II equipped with a FITC filter package to isolate the spectral region between 455–500 nm for excitation and 510–528 nm for emission.

Spectroscopic studies

Absorption measurements were made with a Cary 15 double-beam recording spectrophotometer. Fluorescence spectra were computer-corrected for the wavelength-dependent response of the photon-counting SLM Instruments Model 800 spectrofluorimeter (Urbana, IL) equipped with a Hewlett-Packard data system and software supplied by SLM. The wavelength of maximum emission was assigned to the wavelength at which the first derivative of the corrected fluorescence spectrum was zero. Fluorescence quantum yields (ϕ_f) were calculated from the integrated areas of the corrected fluorescence spectra (20). Fluorescence polarization spectra were obtained as $p = x - 1/x + 1$, by measurement of x, the ratio $I_\parallel/I_\perp$, where $I_\parallel$ and $I_\perp$ are the parallel and perpendicular components of the total fluorescence emission, respectively. Sample temperature was varied with a Neslab Instruments RTE-5 circulating bath and ETP-4RC temperature programmer, with continuous monitoring with a Bailey Instruments Model Bat/8 digital thermometer. Fluorescence lifetimes were measured by the modulation technique at 10 and 30 MHz on an SLM Instruments Model 480 subnanosecond spectrofluorimeter. In organic solvents, the lifetimes measured at these two frequencies were within 0.1 nsec of each other. All spectroscopic measurements were performed at 25°C, unless otherwise noted. Solvents (Burdick and Jackson) contained no detectable fluorescence impurities under these experimental conditions.

DMPC:NBD-CL multilayers

Chloroform solutions of DMPC and NBD-CL were combined in a 50-ml round-bottomed flask and the solvent was evaporated. Tris, 0.025M, pH 7.4, containing 0.15 M NaCl and 0.2 mM EDTA, was added to bring the concentration to 0.2 mg mL^{-1} phospholipid, which contained 1% NBD-CL, w/w. After warming the suspension to 30°C, it was vortexed to produce multilayer liposomes. Fluorescence spectra were measured with constant stirring to maintain thermal equilibrium and suspension of the liposomes. Accurate absorption spectra could not be measured because of the high turbidity of the liposomes and therefore ϕ_f could only be estimated.

23,24-*dinor*-5-Cholen-22-amine-3β-ol

3β-Acetoxy-23,24-*dinor*-5-cholen-22-oyl chloride (21), 5 g (13 mmol), was converted to the corresponding amide by treatment with 60 mL of concentrated NH_4OH for 16 hr at 4°C (22). This treatment also removed the 3β-acetoxy group. After neutralization

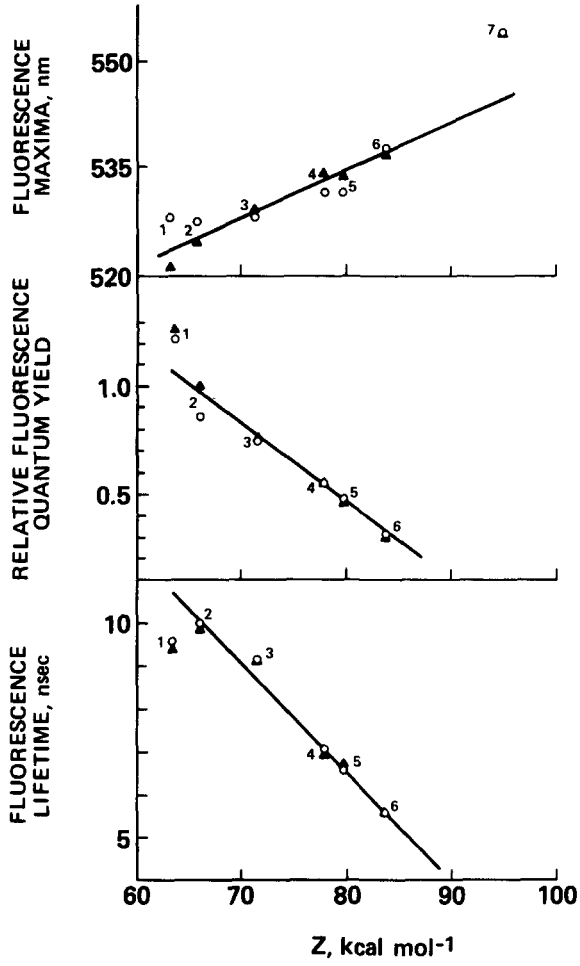

Fig. 2. Effect of solvent polarity on fluorescence properties of NBD-C and NBD-CL. NBD-C (○) and NBD-CL (▲). Solvents were 1) chloroform; 2) acetone; 3) acetonitrile; 4) l-butanol; 5) ethanol; 6) methanol; 7) water.

with 1 M HCl, the product was extracted with $CHCl_3$–CH_3OH 2:1 (v/v) and filtered through charcoal. Infrared analysis revealed the presence of an amide (1680 nm) and ester linkage (1730 nm). The amide, 4.4 g (12.5 mmol), was directly converted to the amine (22) by refluxing overnight with a 10-fold molar excess of $LiAlH_4$ in freshly distilled tetrahydrofuran. The residual $LiAlH_4$ was destroyed by 10 mL each of ethyl acetate and 2M NaOH. The ninhydrin positive product was purified in 40% yield by column chromatography on silica gel (Biorad) with $CHCl_3$–CH_3OH 1:1 (v/v) and was used for synthesis without further characterization.

N-(7-Nitrobenz-2-oxa-1,3-diazole)-23,24-*dinor*-5-cholen-22-amine-3β-ol

23,24-*dinor*-5-Cholen-22-amine-3β-ol, 1.6 g (4.7 mmol), and 4.4 g $NaHCO_3$ were dissolved in 40 mL of 60% ethanol and mixed with 50 mL of ethanol containing 4.4 g (22 mmol) of 4-chloro-7-nitrobenz-2-oxa-1,3-diazole. After a 2-hr incubation at room temperature and neutralization of the reaction mixture with 1 N HCl, the fluorescent product was extracted with chloroform–methanol 2:1 (v/v), dried over sodium sulfate, and purified by column chromatography on silica gel in chloroform–ethyl acetate 9:1 (v/v). The yield based on the amine was 98%; mp 155–160°C. Mass spectra were obtained using a LKB Model 9000 single-focusing instrument with a direct inlet probe; ionizing energy was 70 eV. Mass spectrum: m/e (relative abundance) 494(M^+, 29%), 478(100%), 461(59%), 460(91%), 459(50%), 444(45%), 331(72%), 221(58%), 206(49%). The relative abundances of other ions were less than 5% or m/e was less than 200.

N-(7-Nitrobenz-2-oxa-1,3-diazole)-23,24-*dinor*-5-cholen-22-amine-3β-yl linoleate

The 3β-hydroxyl group was esterified with linoleic anhydride by the method of Patel et al. (23). The sterol, 0.8 g (1.6 mmol), was added to 1.1 g (3.2 mmol) of linoleic anhydride in CH_2Cl_2 containing 0.22 g (1.6 mmol) of 4-pyrrolidinopyridine. The reaction mixture was stirred magnetically for 6 hr at 23°C under N_2. The ester was isolated by column chromatography on silica gel and high pressure liquid chromatography on Prepak-500 silica gel columns (Waters Associates) equilibrated in hexane–ethyl acetate 85:15 (v/v). The yield of ester was 60%. The product contained the theoretical amount of linoleic acid by fatty acid analysis. Mass spectrum: m/e (relative abundance): 577(17%), 443(49%), 432(28%), 314(47%), 313(100%), 298(44%), 283(17%), 281(29%), 256(58%). The molecular ion was not detected.

RESULTS

Fluorescence characterization

As typical examples, the absorption and the uncorrected and corrected fluorescence spectra of NBD-CL in acetone are shown in Fig. 1. The fluorescence properties of both NBD-C and NBD-CL were dependent on the solvent. The fluorescence emission maximum, quantum yield (normalized to unity for NBD-CL in acetone), and fluorescence lifetime varied with solvent polarity Z value (24) (**Fig. 2**). In general, a good linear fit of the data was observed. Differential scanning calorimetry (24) of the ester indicated an endothermic transition centered at 83°C (ΔH 1.46 Kcal mol^{-1}) on heating and on exothermic transition at 59°C (ΔH 1.86 Kcal mol^{-1}) on subsequent cooling (**Fig. 3**). Comparable results

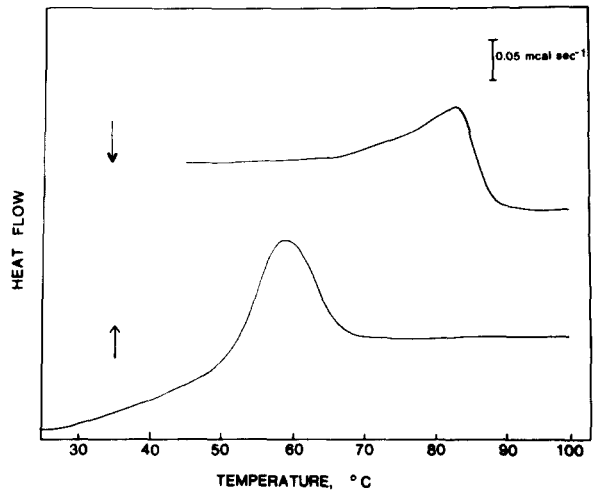

Fig. 3. Differential scanning calorimetry of NBD-CL. NBD-CL, 8.8 mg, was analyzed at a scan rate of 5°C min^{-1} and sensitivity of 0.5 mcal sec^{-1} (25). The arrows indicate the direction of the heat flow.

were obtained with NBD-CL from two separate syntheses. The endothermic transition was confirmed visually on a melting point apparatus. Thin-layer chromatography of material recovered from the analysis indicated no significant decomposition of the compound during repeated scans. Between 5°C and 50°C the thermal dependence of ϕ_f and the fluorescence polarization for r-(NBD-CL)LDL containing either 1 or 100% of the steryl ester content as the fluorophore showed no discontinuities, as anticipated from the calorimetric data.

The fluorescence properties of NBD-CL incorporated into r-(NBD-CL)-LDL were highly concentration dependent. **Table 1** shows that increases in the NBD-CL concentration from 1 to 100% by weight of the steryl ester content decreased the fluorescence intensity, lifetime, and polarization due to self-quenching. The maximum wavelength of absorption and emission were also shifted to longer wavelengths at high NBD-CL concentrations (Table 1). Compared to quinine sulfate in 1 N H_2SO_4 with a ϕ_f of 0.55 (20), NBD-CL in acetone and 1% NBD-CL in r-(NBD-CL)-LDL had relative ϕ_f of 0.14 and 0.04, respectively. Furthermore, in contrast to the results obtained with NBD-CL in isotropic solvents and DMPC liposomes, NBD-CL in r-(NBD-CL)-LDL exhibited multi-exponential fluorescence lifetimes, presumably due to heterogeneous distribution of the cholesteryl ester analog in the reconstituted lipoprotein.

For comparison, the behavior of NBC-CL in a relatively well-defined system of DMPC multilayer liposomes was determined. The temperature profile of DMPC multilayer containing 1% NBD-CL (w/w) exhibited a discontinuity for both ϕ_f and the fluorescence polarization at 24°C, the lipid phase transition of DMPC (**Fig. 4**). Fig. 4 also shows the corrected fluorescence spectrum of NBD-CL in DMPC at 25°C. The maximum fluorescence emission occurred at 535 nm, with an approximate ϕ_f of 0.01–0.05. The fluorescence lifetime of NBD-CL in DMPC liposomes at 25°C was 6.0 nsec.

Specificity as a cholesterol analog

NBD-C was effective as a substrate for LCAT and in suppressing HMG-CoA reductase synthesis in cultured fibroblasts. The suitability of NBD-C as a satisfactory substrate for LCAT was determined with 200 ng of highly purified LCAT (26) and single walled vesicles prepared with 0.12 mM dimyristoylphosphatidylcholine containing 1 mole percent apoA-I and 4 mole percent of either cholesterol or NBD-C (27). After incubation for 30 min at 37°C, 9 pmoles of NBD-CE were formed, as compared to 21 pmoles of cholesteryl ester formed in parallel incubations.

The potencies of the NBD-C and r-(NBD-CL)LDL required to suppress HMG-CoA reductase were compared with cholesterol and LDL, respectively (**Fig. 5**). NBD-C was 60% more effective than cholesterol in reducing mevalonate production (Fig. 5a). Inspection of Fig. 5b reveals that the r-(NBD-CL)LDL inhibited

TABLE 1. Spectroscopic parameters of r-(NBD-CL)LDL

NBD-CL	Wavelength Maxima		ϕ_f	Polarization	Modulation Lifetime	
	Absorbance	Fluorescence			10 MHz	30 MHz
weight percent of steryl ester	*nm*				*nsec*	
1%	458	525	0.039	0.379	9.9	9.3
10%	461	529	0.021	0.292	8.3	7.4
50%	462	536	0.007	0.207	6.1	5.1
100%	465	545	0.001	0.144	3.5	2.8

r-(NBD-CL)LDL, prepared as described in Methods, was examined at 25°C. Quantum yields are those relative to $\phi_f = 0.55$ for quinine sulfate in 1N H_2SO_4.

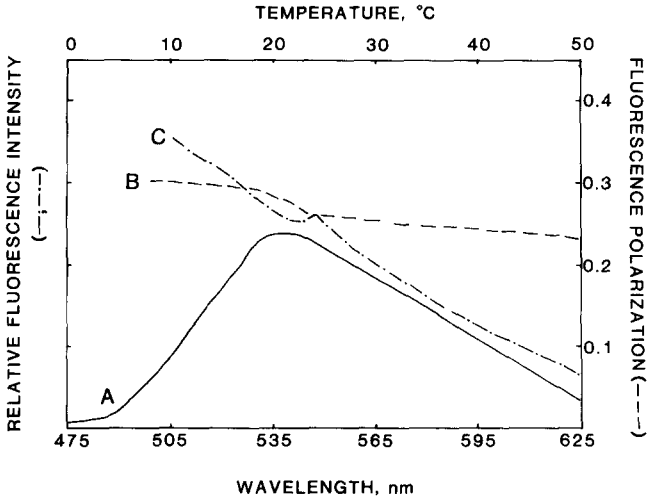

Fig. 4. The fluorescence properties of NBD-CL in DMPC multilayers. A, (——) is the wavelength corrected emission spectrum; B, (– – –) is the thermal dependence of the fluorescence polarization and C, (–·–·–) is the thermal dependence of the fluorescence emission monitored at 530 nm. The DMPC contained 1% NBD-CL (w/w).

HMG-CoA reductase activity, but less effectively than did LDL. In separate experiments, r-(CO)LDL was about half as effective as native LDL at the same protein concentration in suppressing HMG-CoA reductase.

Interaction of r-(NBD-CL)LDL with fibroblasts

After lysosomal accumulation, LDL cholesteryl esters are hydrolyzed to cholesterol, a portion of which is re-esterified (3). After an 18-hr incubation of r-(NBD-CL)LDL with fibroblasts, two fluorescent products were extracted. Approximately 97% of the fluorescent material extracted from the cells comigrated with NBD-C; the second fluorescent compound comigrated with NBD-CL.

Incubation of r-(NBD-CL)LDL with human skin fibroblasts at 37°C for time intervals up to 48 hr did not change the cellular morphology at the light microscope level. Viability, as assessed by trypan blue exclusion, was unaltered. Subsequent subcultivation revealed that the growth rate was normal. The uncorrected fluorescence emission spectrum of NBD in the cells was identical to that in either LDL or in $CHCl_3$–CH_3OH 2:1 (v/v).

The similarity of the fluorescence properties of NBD to FITC allowed direct visualization of cellular accumulation of r-(NBD-CL)LDL by conventional fluorescence microscopy (**Fig. 6a**). At 37°C after 2 hr of incubation with r-(NBD-CL)LDL, the accumulation of fluorescence was characterized by a perinuclear distribution similar to that of lysosomes (9, 28). Despite the large differences in quantum yield between r-(NBD-CL)-LDL containing either 1 or 100 weight percent of the steryl ester as NBD-CL, little difference was noted in fluorescent intensity of cells incubated with such particles as judged by fluorescent microscopy. Cells incubated with r-(NBD-CL)-LDL that contained only 1% NBD-CL did have a somewhat lower general cytoplasmic fluorescence, presumably due to a reduction in the amount of free NBD-C being released from the lysosomes. Furthermore, the distribution of fluorescence was similar to that of normal LDL as revealed by anti-LDL immunofluorescence (Fig. 6b). No cellular fluorescence as the result of surface binding of r-(NBD-CL)LDL could be directly visualized at 4°C, although surface accumulation of these particles was demonstrated by anti-apoLDL immunofluorescence. Moreover, no intracellular fluorescence could be observed in the following experiment. r-(NBD-CL)-LDL was bound at 4°C to cells and the unbound lipo-

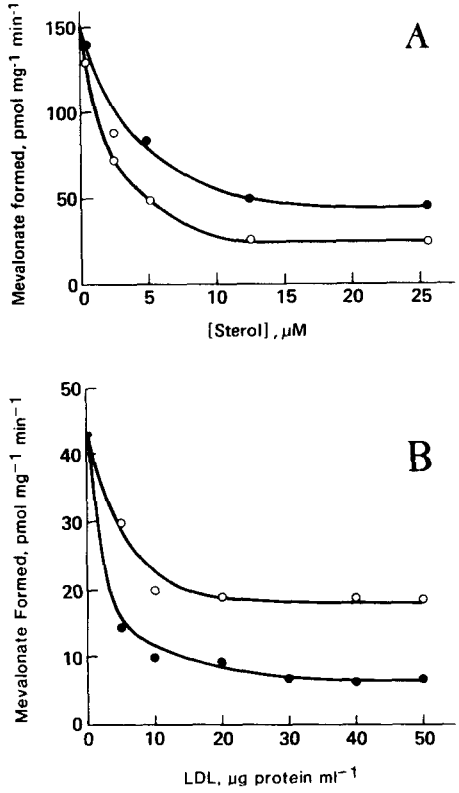

Fig. 5. Suppression of HMG-CoA reductase by NBD-C (○ —— ○), and cholesterol (● —— ●), r-(NBD-CL)LDL (○ —— ○), and LDL (● —— ●). Fibroblasts were plated in triplicate in 2 ml of lipoprotein-deficient medium. In panel A, the indicated amount of sterol in ethanol was added. In panel B, the indicated amount of LDL or r-(NBD-CL)LDL was added. The mass ratio of NBD-CL to protein in this preparation of r-(NBD-CL)LDL was 1.1:1.0; whereas in LDL, the cholesteryl ester:protein ratio was 1.3:1.0. After 16 hr, the cells were washed and harvested as described (11). Each data point is the average of triplicate dishes, each assayed in duplicate, which differed less than 10%.

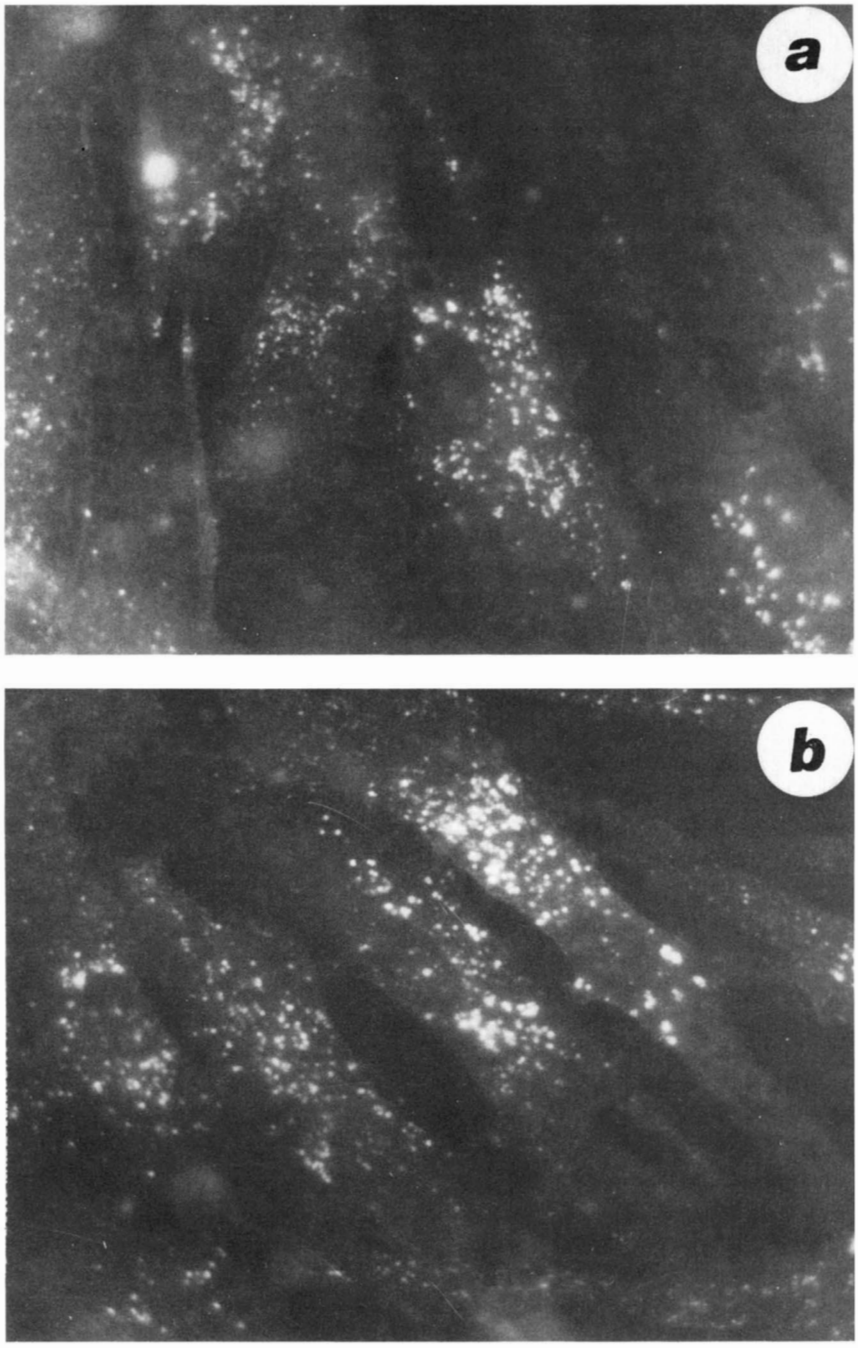

Fig. 6. Intracellular distribution of r-(NBD-CL)LDL fluorescence, A) and FITC anti-apoB immunofluorescence, B). Magnification was 400×.

proteins were removed with albumin washes. The temperature was then raised to 37°C for 1 hr so that internalization of surface bound LDL could proceed. Thus, the fluorescence associated with the limited amount of LDL bound to the cell surface at 4°C was below the sensitivity limit of the microscope. Uptake of fluorescent LDL after 2 hr was a specific process, since LDL receptor-negative cells did not accumulate r-(NBD-CL)LDL fluorescence, and r-(NBD-CL)LDL uptake in normal fibroblasts was prevented by a 25-fold excess of LDL.

Visualization of r-(NBD-CL)LDL by fluorescence microscopy suggested that, with more sensitive instrumentation, fluorescence could be used to quantify lipoprotein-cell interactions. As demonstrated in the inset of **Fig. 7,** there was a linear relationship be-

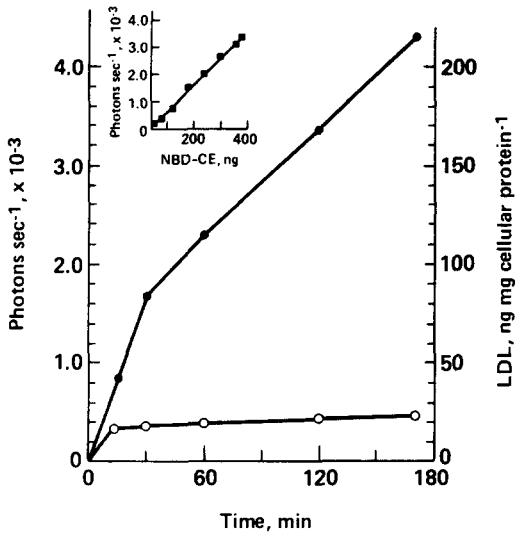

Fig. 7. Effect of time of incubation on cell associated NBD fluorescence. At the indicated times, NBD fluorescence was quantified by single photon counting as described in Materials and Methods. (○ —— ○), surface bound and (● —— ●), intracellular fluorescence.

tween cellular NBD-CL content and fluorescence intensity. When cells were incubated with r-(NBD-CL)LDL and the total fluorescence was quantified after extraction with $CHCl_3$–Ch_3OH 2:1 (v/v), a time-dependent increase in fluorescence was observed. To determine that values for binding and endocytosis by fluorescence quantitation were comparable to values obtained with [^{125}I]iodo-LDL, a double label experiment was performed using r-(NBD-CL) [^{125}I]-iodo-LDL. Following incubation of r-(NBD-CL) [^{125}I]-iodo-LDL with fibroblasts in triplicate plates for 6 hr, values for surface-bound LDL were 24.8 ng/mg and 32.6 ng/mg for fluorescence and isotopic determinations, respectively. The amount of LDL endocytosed was 1387 ng/mg by total intracellular fluorescence and 1552 ng/mg by isotopic evaluation, which was the sum of intracellular [^{125}I]iodo-LDL and extracellular degradation products. The interactions of r-[^{125}I]iodo-LDL containing between 1 and 100 weight percent steryl ester as NBD-CL and cells were identical.

DISCUSSION

The preparation of the cholesterol analog described in this report is straightforward and yields a suitably fluorescent sterol. The data in Fig. 4 show that, in a simple phosphatidylcholine system, the fluorescent steryl ester is able to detect the thermally induced phase transition of DMPC. Furthermore, the NBD-CL fluorescence lifetime and λ_{max} data indicate that, on the basis of the data in Fig. 2, the interior of the phospholipid bilayer is fairly polar. This finding corroborates the previous report of studies with N-(2-naphthyl)-23,24-*dinor*-5-cholen-22-amine-3β-ol (7), another fluorescent cholesterol analog. Since the fluorescent sterol and steryl ester containing the 7-nitrobenz-2-oxa-1,3-diazole group are significantly less susceptible than the naphthyl-containing analogs to photodecomposition, they should also be useful for studies of the interior microenvironment of cellular membranes. Although the NBD-C is more polar than cholesterol and has the same mobility as 25-hydroxycholesterol in several thin-layer chromatographic solvent systems, NBD-C appears to be a reasonable cholesterol analog, based on its 40% effectiveness as an acyl chain acceptor in the reaction catalyzed by LCAT. This level of activity is comparable to other sterols with modified side chains, such as camposterol, β-sitosterol, desmosterol, stigmasterol, and N-(2-naphthyl)-23,24-*dinor*-5-cholen-22-amine-3β-ol, which are less active as substrates for LCAT (7, 29). NBD-C also resembles 25-hydroxycholesterol in its enhanced suppression of HMG-CoA reductase (30, 31). The lower effectiveness of r-(NBD-CL)LDL, as compared to LDL, in suppressing HMG-CoA reductase is intriguing in view of the greater potency of NBD-C added directly. The differences between LDL and r-(NBD-CL)LDL may be due to a greater amount of cholesteryl ester in native LDL. In addition, the conflicting reports (32, 33) on the physicochemical properties of LDL reconstituted by the procedure of Kreiger et al. (12) leave open the possibility that some of these differences in biological activities may be associated with the relatively undefined structure of the reconstituted lipoprotein. It appears, however, that intracellular distribution of cholesterol originating from lysosomal breakdown of LDL is more efficient than the release of NBD-C from r-(NBD-CL)LDL.

Replacement of the native cholesteryl ester of LDL with NBD-C linoleate gives a fluorescent LDL particle with which LDL-mediated sterol accumulation in fibroblasts can be visualized, as previously demonstrated with r-(PMCA oleate)LDL (8, 9). The inability to detect the fluorescence of surface-bound LDL particles at 4°C is probably due to the combined effects of the relatively low extinction coefficient of NBD-CL (20,000) as compared to fluorescein (66,000) or pyrene (50,000), the low quantum yield of the fluorophore in r(NBD-CL)LDL, and the limited sensitivity of the microscopy system. This interpretation is supported by the recent studies of Anderson, Goldstein, and Brown (9) who reported

the detection of the fluorescence of r-(PMCA oleate)-LDL bound to the fibroblast plasma membrane at 4°C. Highly sensitive video intensification microscopy (34) should circumvent these experimental difficulties and permit visualization of the surface distribution of LDL and its subsequent endocytosis.

The availability of fluorescent r-(NBD-CL)LDL should greatly facilitate studies of LDL metabolism in various cell systems. The isolation of cells deficient in LDL receptors has been described in a recent report by Goldstein et al. (35). Cells containing high affinity LDL receptors could be separated from receptor negative cells by the use of r-LDL containing dioleoylfluorescein and a fluorescence-activated cell sorter. NBD-CL has major advantages over dioleoylfluorescein in that the rate of passive cholesteryl ester partitioning from lipoproteins is essentially nil and there is very little background fluorescence associated with r(NBD-CL)LDL, thereby obviating the necessity for preliminary bleaching of samples prior to measurement. Experiments in this laboratory[1] have demonstrated that r-(NBD-CL)LDL bound to erythrocytes coated with anti-apoB are readily separated by a fluorescence-activated cell sorter. Moreover, lymphocytes incubated with r-(NBD-CL)LDL could be distinguished with this instrument from lymphocytes that had been incubated separately with excess unlabeled LDL. In addition, the ester probe provides a non-exchangeable marker for visualizing fusion of phospholipid vesicles with fibroblasts. Finally, NBD-C and NBD-CL can be of value in other areas of membrane research. The sensitivity of the probes to solvent polarity suggests that they may be suitable for monitoring the microenvironment of cholesterol in the cell membranes.

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[1] Via, D. P., and L. C. Smith. Unpublished observations.

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