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THE SYNTHESIS AND PROPERTIES OF A FUNCTIONAL FLUORESCENT CHOLESTEROL ANALOG

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The synthesis of a new fluorescent cholesterol analog is described. The analog contains a cholesterol nucleus attached via a hydrophilic spacer to *N*-4-nitrobenzo-2-oxa-1,3-diazole. Since the cholesterol moiety is not perturbed this molecule probably interacts with lipid bilayers in much the same way as cholesterol itself does. The compound can be readily incorporated into small unilamellar vesicles by sonicating a mixture of it with egg yolk phosphatidylcholine in a buffer. Furthermore, the analog can be incorporated into preformed membranes either by exchange from vesicles containing the analog or by uptake from sonicated micelles of the analog. Thus this analog shows potential as a useful tool for studying the interactions of cholesterol with cell membranes.

Introduction

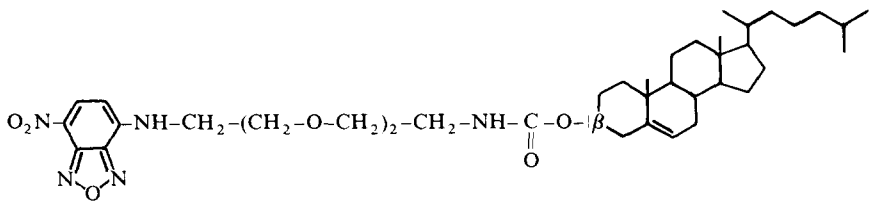
Although cholesterol is an important constituent of most eucaryotic membranes, surprisingly little is known about its role in membrane function. A complete understanding of cholesterol's functional role would have to include knowledge of its distribution in membranes as well as its rate of lateral moblity. To these ends a fluorescent analog of cholesterol which preserved the steroidal skeleton intact and which could be incorporated into membranes would be useful. Previously, we had shown that cholesterol analogs such as compound 5 could be functionally incorporated into vesicles in much the same way as cholesterol itself [1–3]. The derivatives, compounds 4 and 5 *, distribute evenly on both sides of the bilayer, they render egg yolk phosphatidylcholine based sonicated vesicles less permeable to small molecules, they have an effect on the order parameter similar to that of cholesterol, and finally the ^{13}C-NMR signals of the cholesterol moiety of the synthetic glycolipids disappear when it is membrane bound [1–3]. The water soluble nature of the side chain also allows for the facile incorporation of these analogs into cells [4]. In this article, we show that methodologies utilized for the preparation ofthe synthetic glycolipids can be applied to the preparation of a new fluorescent cholesterol analog 4 which is also expected to interact with the lipid bilayer in a way similar to or identical to that of cholesterol itself. Furthermore, the interactions of the fluorescent analog with small unilamellar vesicular membranes is characterized.

* For structural formula see opposite page.

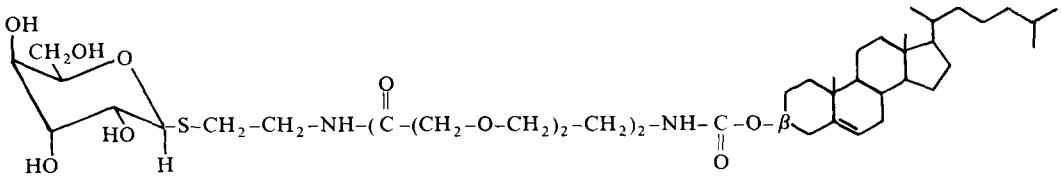
Abbreviation: NBD, 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Materials and Methods

Ricin, the β-galactosyl-binding agglutinin from *Ricinus communis*, was obtained from Boehringer Mannheim Inc. Egg phosphatidylcholine was prepared and purified according to the method of Litman [5]. The phospholipid concentration was



Compound 4



Compound 5

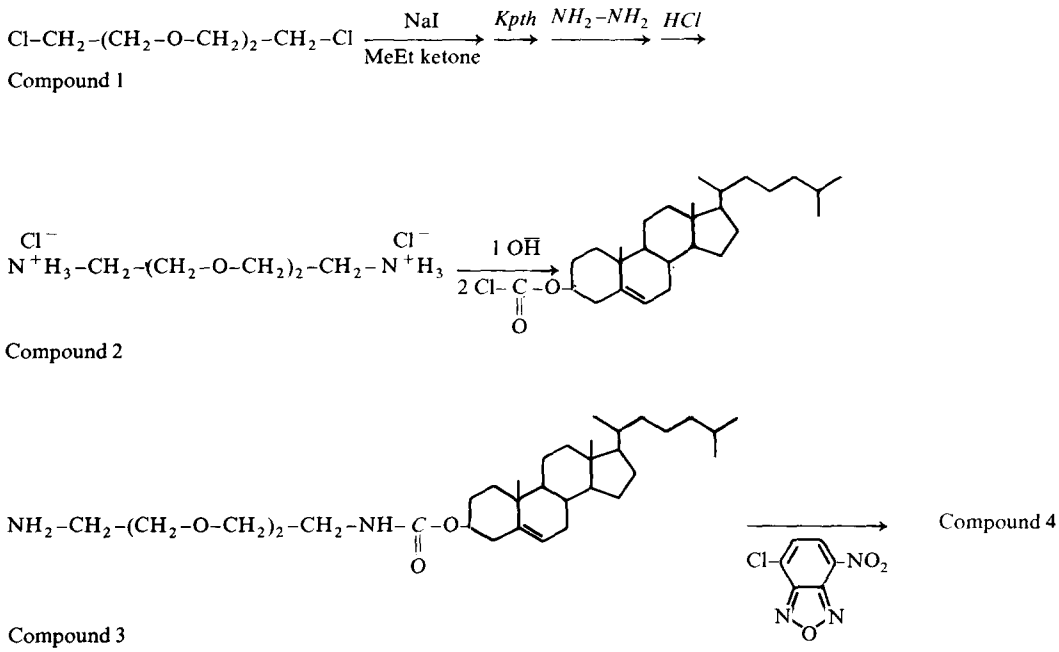
determined as inorganic phosphate after ashing and acid hydrolysis [6]. The phospholipid was dissolved in benzene at a concentration of 40 μmol/ml and stored at −70°C under an atmosphere of nitrogen. The purity of the preparation was checked routinely by thin-layer chromatography (silicic acid; $CHCl_3/CH_3OH/H_2O$, 65:25:4, v/v. [7-^{3}H]Cholesterol was purchased from New England Nuclear Inc. Lactosylceramide (*N*-palmitoyldihydrolactocerebroside) was purchased from Miles Laboratories, Elkhart, Ind. Cholesterol chloroformate was a product of the Sigma Chemical Co. 1,2-Bis(2-chloroethyoxy)ethane and 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD) were products of Eastman Organics. The dichloro compound required careful distillation before use. Potassium phthalimide was a product of the Aldrich Chemical Co. Dimethylformamide, triethylamine, methylethylketone, isopropanol, and sodium iodide were products of the Fisher Chemical Co. Chemical analyses were performed by Galbraith Labs., Knoxville, TN.

Syntheses

The synthesis of the fluorescent cholesterol analog 4 was carried out by the following sequence of steps (see Scheme I).

(A) 1,2-Bis(2-aminoethoxy)ethane dihydrochloride compound 2, hydrophilic spacer). Sodium iodide (97.4 g, 0.65 mol) was dissolved with stirring in 350 ml dry methylethyl ketone in a 1 litre single necked flask; 1,2-bis(2-chloroethoxy)ethane (46.5 g, 0.25 mol), previously distilled, was added and the mixture was stirred under reflux for 19 h. The mixture was allowed to cool to room temperature and filtered to remove the salts. Methylene chloride (200 ml) was added and the solution was extracted with a solution of sodium bisulfite (20 g in 50 ml H_2O), followed by a mixture of sodium bicarbonate (20 ml of 0.5 M) and saturated sodium chloride (50 ml), and finally washed three times with 100-ml portions of the saturated sodium chloride solution.

The organic extract was dried over anhydrous sodium sulfate, filtered and evaporated to dryness to yield 88.7 g (0.24 mol) of the 1,2-bis(2-iodoethoxy)ethane. Without further purification the diiodo compound 74 g (0.2 mol) was dissolved in 200 ml dimethylformamide (distilled over CaH_2) and 75 g (0.41 mol) potassium phthalimide was added. The solution was stirred at 95° for 19 h. The yellow solution was allowed to cool to room temperature and then poured into 1.5 l of ice water and filtered. The light yellow solid product was washed with 500 ml H_2O, 500 ml ethanol and dried overnight to yield 58 g (0.144 mol) of the diphthalimide adduct as a white powder. Without further purification this material was dissolved in 600 ml methanol containing 9.6 g (0.3 mol) anhydrous hydrazine and refluxed overnight. The



Scheme I. Synthesis of compound 4. Kpth, potassium phthalimide.

solution was evaporated, 200 ml H_2O was added and the solution was then filtered, and the filtrate was acidified to pH = 2 with concentrated HCl. The precipitate was filtered off and the filtrate was evaporated to a yellow oil which was crystallized, after carbon black treatment, from ethanol to afford the dihydrochloride salt of the diamine as a white crystalline solid. Yield: 12 g (0.056 mol), m.p. = 127–128°C.

Anal. calc. for $C_6H_{18}CL_2N_2O_2$: %C 32.73; %H 8.18; %N 12.73.

Found: %C 32.69; %H 8.22; %N 12.87

(B) 8-(N-Cholesterylcarbamoylamino)-3,6-dioxaoctylamine (compound 3). The free base 1.3 g (8.8 mmol) was prepared from the diamine hydrochloride described above and was dissolved in 25 ml isopropanol along with 0.89 g triethylamine. With stirring, 1.35 g (3 mmol) of β-cholesterol chloroformate was added in 3 ml benzene over 1 h. The suspensions was stirred overnight. The suspension was filtered and the filtrate was taken to dryness. The oily precipitate was dissolved in methylene chloride and extracted once with 10% Na_2CO_3 and five times with saturated sodium chloride. The organic layer was dried over anhydrous sodium sulfate, taken to dryness, and extracted four times with 5 ml cold methanol. The bis-cholesterol adduct hyproduct (2.2 g) is sparingly soluble in methanol and precipitates. The product was finally obtained as a ninhydrin-positive colorless oil (0.8 g, 1.42 mmol) after evaporation of the methanol.

(C) 8-(N-Cholesterylcarbamoylamino)-3,6-dioxaoctyl-1-amino(N-4-nitrobenzo-2-oxa-1,3-diazole) (compound 4). The cholesterylamine 0.75 g (1.34 mmol) prepared above was dissolved in 2.5 ml ethanol in a 50-ml three-necked flask wrapped with aluminum foil and flushed with dry N_2. Triethylamine (0.2 ml) was added with stirring at room temperature. An ethanolic solution of NBD (0.267 g NBD/5 ml ethanol) was dropped in over 0.5 h with stirring. The solution was allowed to stir at room temperature for an additional hour under N_2. The ethanol was removed in dim light under N_2 by evaporation. The dark solid residue was chromatographed on EM silica gel 60. A step gradient of chloroform (to remove the unreacted NBD) followed by 5% methanol/chloroform was

used. The product eluted with the latter solvent system. After evaporation of the solvent, the compound was recrystallized from ethanol to yield 0.46 g (0.63 mmol) of the product as a yellow crystalline solid m.p. 129–134°C (dec.); the NMR, infrared, ultraviolet, and fluorescence spectra of the compound were entirely consistent with the assigned structure.

Anal. calc.: %C 66.39; %H 8.29; %N 9.68.

Found: %C 66.31; %H 8.33; %N 9.50.

The compound was stored at −20° as a solid until needed.

Small unilamellar vesicles

The small unilamellar vesicles used in these studies were prepared from the cholesterol derivatives and egg yolk phosphatidylcholine by a method identical to that published by Barenholz [7]. The phosphatidylcholine in chloroform and the fluorescent probe in methanol were dried down in a flask in vacuo overnight. The buffer (50 mM Tris (pH 7.4) plus 140 mM NaCl) was added and the mixture was flushed with argon, tightly capped and wrapped in aluminum foil. The mixture was then sonicated with a Laboratory Supplies G112SP1G bath sonicator at 5°C until the mixture clarified. The vesicles were centrifuged at 55000 × *g* for 16 h. The upper two thirds of the centrifuge tube was collected as the vesicles. The phospholipid concentrations were determined by the published procedure [6].

Fluorescence measurements

The fluorescence measurements reported in this article were performed on a Perkin-Elmer model 512 double-beam spectrometer at 25°C. Where fluorescent vesicles were studied the reference sample contained the same concentration of vesicles (fluorescent probe) as in the sample beam to control for light scattering effects.

Agglutination assays

The vesicles made up in the already described buffer. Ricin was added and the increase in absorbance at 360 nm was followed with time by the published procedure [1–3]. This assay has been shown to be linearly related to the amount of phospholipid precipitated and can thus serve as a quantitative measure of the amount of aggregation [1–3]. The measurements were performed on a Gilford model 240 spectrophotometer.

Results

(1) Synthesis and characterization of compound 4

The NBD analog was prepared containing a hydrophilic spacer group in order to increase its

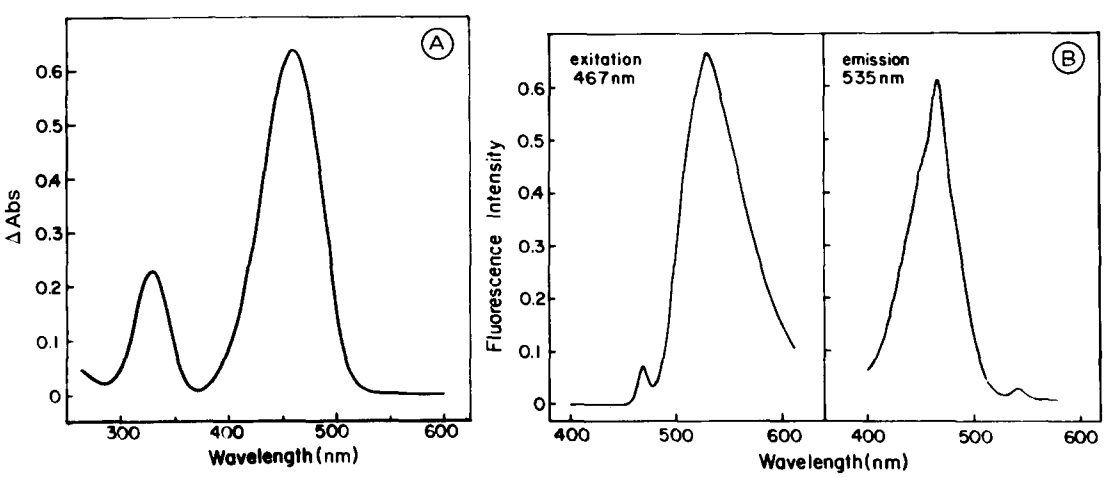


Fig. 1. The absorption and fluorescence spectra of compound 4. In (A) the absorption spectrum of the pure compound at a concentration of $3.8 \cdot 10^{-5}$ M in ethanol was measured in 10 mm cells. The measured $\epsilon^{mM}_{460nm} = 19.5$. In (B) the fluorescence excitation and emission spectra of a $3.5 \cdot 10^{-5}$ M solution of the compound was determined in ethanol and are uncorrected. In the measurements reported in the figure the sensitivity level of the fluorometer was set at 3.

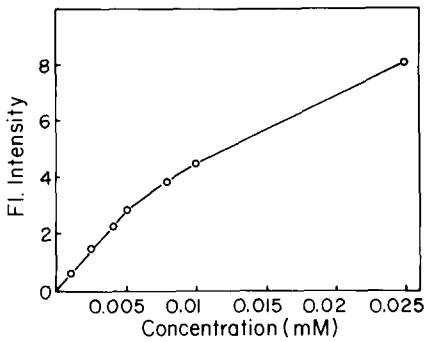


Fig. 2. Fluorescence measurement of varying concentrations of compound 4 in ethanol. Ethanolic solutions of compound 4 at the indicated concentrations were made up and their emission intensities at 535 nm were recorded exciting at 470 nm. A sensitivity setting of 0.3 was used on the spectrometer.

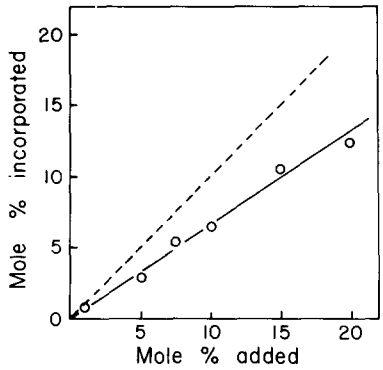


Fig. 3. Incorporation of compound 4 into small unilamellar vesicles. The vesicles were prepared from compound 4 and phosphatidylcholine by the usual methods, starting with different concentrations of compound 4. The vesicles containing compound 4 were purified by centrifugation. The phospholipid concentrations and fluorescence intensities of the vesicles were masured. The latter was determined by dissolving small aliquots of the vesicles in ethanol and comparing the fluorescence emission at 535 nm with excitation at 470 nm with a standard curve of compound 4 in ethanol. O, determined amount of compound 4 in the vesicles. In the buffer, the vesicles containing compound 4 emit at 540 nm and excite at 470 nm.

solubility in water. The synthesis was carried out as described in Methods. The product was characterized by both spectroscopic and analytical data. The absorption and fluorescence spectra of the pure compound in ethanol are shown in Fig. 1. In Fig. 2 a plot is given of the relative fluorescence intensity of varying concentrations of the probe in ethanol.

(2) Incorporation of compound 4 into small unilamellar vesicles and their characterization

Varying amounts of the analog 4 were dissolved in chloroform along with pure phosphatidylcholine. After drying, the mixture was suspended in buffer and sonicated [7]. The resultant small unilamellar vesicles were then purified and the fluorescence emission and excitation spectra were determined. The λ_{max} for excitation is 470 nm and that for emission is 540 nm. When these vesicles were dissolved in ethanol and the fluorescence spectra were again recorded only approximately a 13% increase was measured. Thus, the quantum yield of fluorescence of the analog is roughly the same in the membrane as it is in pure ethanol. To quantitate the amount of compound 4 incorporated into the membrane, the small unilamellar vesicles were dissolved in ethanol and the fluorescence intensities of the resultant solutions were measured (Fig. 3). These experiments show that the amount of compound 4 incorporated in the small unilamellar vesicles linearly increases with increasing amounts of compound 4, but only about 50% of the added compound is actually incorporated in the small unilamellar vesicles. As with other *O*-alkylated cholesterol analogs, only about 15% of compound 4 can be maximally incorporated in the small unilamellar vesicles [1–3]. The resulting small unilamellar vesicles were sized on a Sepharose 4B column (Fig. 4). The peak phospholipid fractions co-eluted with the peak of fluorescence. An average mean diameter of approx. 280 Å was obtained for these small unilamellar vesicles by comparing their elution rate with that of latex beads of known dimensions.

The experiments described above suggest that the fluorescent analog 4 is associated with the vesicles as expected. However, further evidence was required to unequivocally show that compound 4 was incorporated into the vesicles. To these ends, the vesicles were prepared containing glycolipid 5 and 4. Small unilamellar vesicles containing compound 5 are aggregated by the β-galactoside binding lectin from *Ricinis communis* (ricin) [1–3]. Furthermore, the rates of aggregation are quite sensitive to the state of the membranes' organization [1–3]. It compound 4 is indeed incorporated into the small unilamellar vesicles like

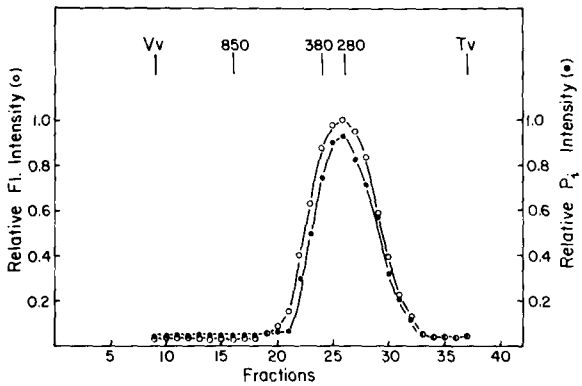
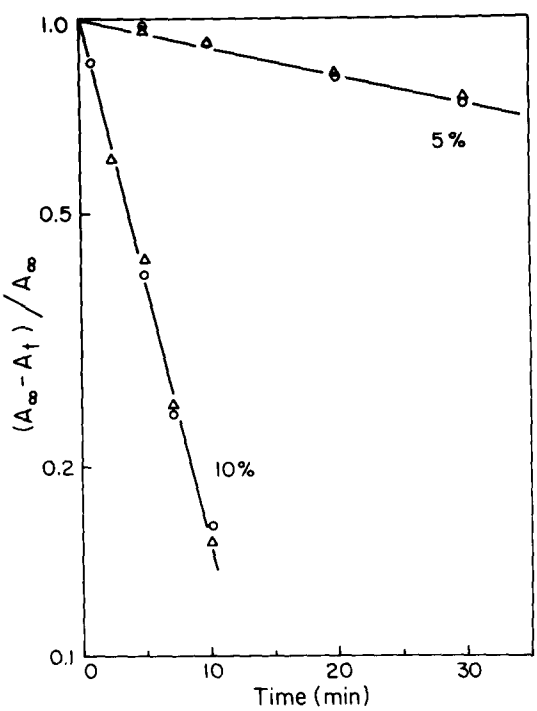


Fig. 4. Size characterization of small unilamellar vesicles. A Sepharose 2B column (20×0.75 mm) was prepared and equilibrated with Tris-HCl (50 mM, pH 7.4) and 140 mM NaCl along with 1% Triton X-100. The void volume (Vv) was determined using 2250 Å latex particles. The total volume (Tv) was determined with [^{14}C]glutamate. Other standards used were 850 Å and 380 Å latex particles. After standardization, the column was washed with 10 volumes of the buffer to remove the detergent. Vesicles containing 10% of compound 4 were prepared. 200 μl of 3 μmol (P_i/ml of the vesicles were loaded on the column and eluted with the buffer. Fractions (1 ml) were collected and the phosphate concentrations and fluorescence intensities in ethanol were determined. The average diameter of the vesicles was taken to be 280 Å.



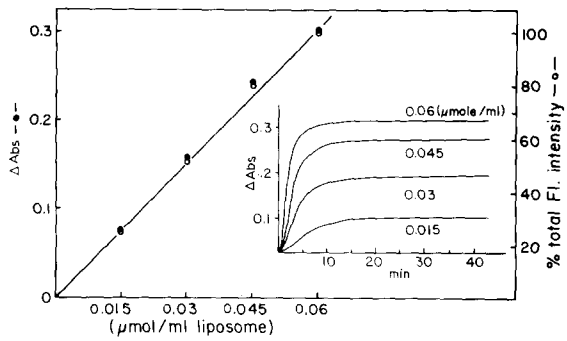


Fig. 6. The same vesicles contain compounds 4 and 5. Vesicles containing 10% of compound 5 and 5% of compound 4 were prepared. The vesicles were made up to a final concentration of 0.015 μmol/ml, 0.03 μmol/ml, 0.045 μmol/ml, and 0.06 μmol/ml phospholipid, respectively, in the usual Tris buffer at 10°C. each sample was charged with 200 μg/ml ricin and the rate and extent of vesicle aggregation was followed at 360 nm as shown above (insert). The suspensions were centrifuged and the precipitates were assayed for fluorescence after dissolution in ethanol and removal of the precipitated protein. In previous experiments, we had shown that the amount of phospholipid precipitated under these conditions was directly proportional o the recorded absorbance (Abs) [1–3].

cholesterol, then the small unilamellar vesicles prepared from compounds 4 and 5 should be aggregated by ricin at the same rate as those prepared with the same concentration of compound 5 and cholesterol. The kinetics (initial rates) of the ricin mediated aggregations of small unilamellar vesicles containing compounds 5 and 4 at different concentrations is shown in Fig. 5. By way of comparison, the rates of aggregation of the small unilamellar vesicles containing only compound 5 and cholesterol are included. As can be seen from Fig. 5, the rates of aggregation are virtually identical. It should be noted that sterol, in and of itself, has very little, if any, effect on the rates of aggregation [1–3]. That the aggregated small unilamellar vesicles contained compound 4 is shown in Fig. 6. By varying the phospholipid concentrations

Fig. 5. Ricin-mediated aggregation of vesicles containing compounds 4 and 5. Vesicles were prepared containing 5% and 10% of compound 5 and either 5% cholesterol or 5% compound 4. The rates of aggregation of these vesicles with 100 μg/ml ricin at a phospholipid concentration of 0.06 μmol/ml were determined according to the published procedure [1–3]. ○, vesicles containing compound 4; △, vesicles containing cholesterol.

the amount of small unilamellar vesicle aggregation at a single ricin concentration can be varied [1–3]. The amount aggregated is directly proportional to the concentration of phospholipid [1–3]. In Fig. 6 small unilamellar vesicles containing compounds 4 and 5 were aggregated to different extents as determined by the amplitude of light scattering measured at 360 nm. The precipitated phospholipid was washed followed by decantation of the washing buffer and final dissolution of the pellet in ethanol. As shown in Fig. 6, the fluorescence intensity of the solution was directly proportional o the amount of phospholipid aggregated by the ricin. These experiments uneqivocally demonstrated that the fluorescent cholesterol analog 4 is directly incorporated in the small unilamellar vesicle membrane.

(3) Exchange of compound 4 into membranes

If the new fluorescent cholesterol analog 4 is to be fully useful, it must be capable of being incorporated into preformed membranes by an exchange process. For this reason, evidence is presented here showing that compound 4 can, in fact, be incorporated into small unilamellar vesicle membranes. Sonicated suspensions of compound 4 in buffer are non-fluorescent due to self-quenching. When phosphatidylcholine-based small unilamellar vesicles are added to this sonicated suspension the fluorescence of the solution gradually increases. It could be shown that this fluorescence is associated with the vesicles by gel filtration (Fig. 7). These experiments show that there can be an excange of the fluorescent analog from the micelle stage into the membrane. In order to show that exchange of the analog 4 can occur from membrane to membrane, the experiments shown in Fig. 8 were performed. Small unilamellar vesicles were prepared containing 10% lactosylceramide plus either 5% colesterol or 5% compound 4. The small unilamellar vesicles were mixed and at various time points the agglutinin from *Ricinus communis* (ricin) was added to aggregate the ceramide containing vesicles. In these experiments the vesicles containing the non-exchangable ceramide act as acceptors, whereas those bearing compound 4 are donors. That no exchange of lactosylceramide occurs under the conditions of these experiments has been amply demonstrated

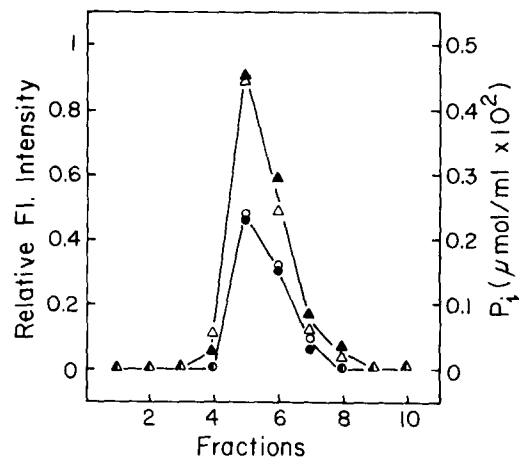


Fig. 7. Incorporation of sonicated suspensions of compound 4 into small unilamellar vesicles. The fluorescent analog 4 (0.5 μmol/ml) in the usual Tris buffer was sonicated until clarified. This served as the stock solution of sonicated fluorochrome. The sonicated suspension itself, however, was not fluorescent due to self-quenching. Vesicles were also prepared at a concentration of 1.9 μmol/ml phospholipid in buffer. The micellelar solution (50 μl), was mixed with either 50 μl (+50 μl buffer) or 100 μl of the solution of vesicles. The solutions were allowed tos tir for 3 h at 25°C. The samples were passed through a short column of Sepharose 2B (4 ml) and eluted with the buffer. Ten 1-ml fractions were collected and the fluorescence intensities and phosphate concentrations of the fractions were determined. The fluorescence was measured by exciting at 470 nm and measuring emission at 540 nm. △, fluorescence intensity and ▲, phosphate concentration of the more concentrated vesicular solutions; ○, the fluorescence intensity and ●, phosphate concentration of the less concentrated solutions.

[8,9]. The aggregated vesicles were assayed for fluorescence (Fig. 8). The fluorescence associated with these vesicles is assumed to have occurred via exchange of compound 4 from one vesicle to another, since ricin does not aggregate vesicles in the absence of incorporated β-galactoside. For comparion's sake, in Fig. 8 the exchange of [^{3}H]cholesterol from one small unilamellar vesicle to another is also shown. This method was adapted from the one used by Dawidowicz to measure rates of intermembrane cholesterol exchange [9]. It should be noted that our exchange experiments were not done under conditions where rate constants for the exchange process can be measured, because of back reactions. However, the experiments with vesicles containing compound 4 make it clear that intermembrane exchange of the analog

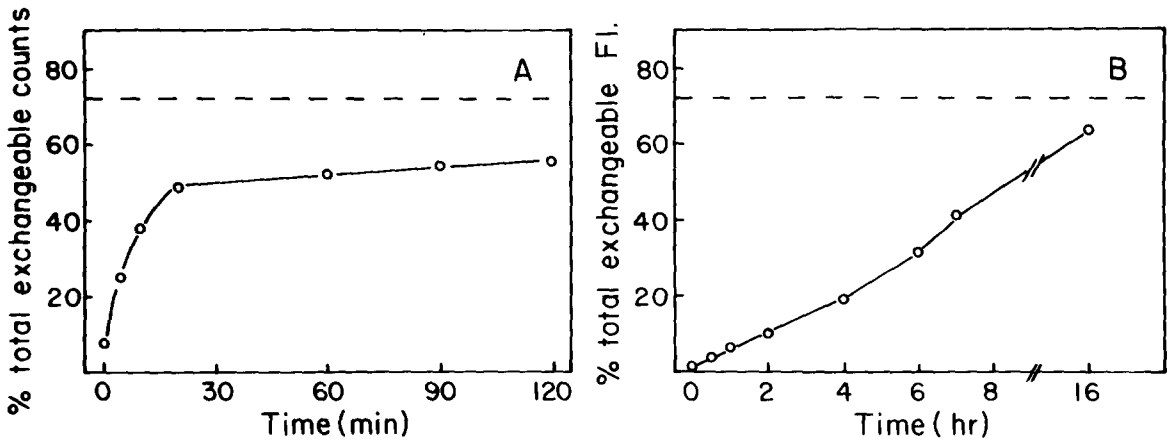


Fig. 8. The intermembraneous exchange of compound 4. Small unilamellar vesicles were prepared containing 5% of compound 4, 5% [7-^{3}H]cholesterol (spec. act. 20 μCi/μmol) and 5% cholesterol plus 10% lactosylceraide. In separate experiments the lactosylceramide-containing vesicles (2 ml, 0.5 μmol/ml) were mixed with vesicles containing either 5% of compound 4 (0.4 ml, 0.6 μmol/ml) or 5% [7-^{3}H]cholesterol (0.4 ml, 0.6 μmol/ml) at 37°C. At the indicated time points, aliquots of the mixtures were removed and added to 1 ml of 200 μg/ml ricin at 10°C. As controls in both cases the donor vesicles were added directly to the ricin solution. The aggregation reactions were allowed to proceed for 30 min at which time the aggregates were centrifuged. The pellets and supernatant were assayed for ^{3}H or fluorescence after dissolution in ethanol. The controls, which contained $<5\%$ of the ^{3}H or fluorescence, were subtracted in every case. In the above graph (A) the rate of [7-^{3}H]cholesterol exchange (max possible incorporation cpm) is given along with the rate of compound 4 exchange (B), expressed as the % of total possible fluorescence incorporated. In both cases the dotted lines refer to the maximum amount of [^{3}H]cholesterol or compound 4 which could be incorporated into the acceptor vesicle assuming total equilibration of the cholesterol pools; that is, it is assumed that the relative amount of the compounds ending up in the donor or acceptor vesicles is solely determined by the donor and acceptor phospholipid concentrations.

occurs, albeit at a rate considerably slower than that for cholesterol itself under the conditions of the experiments.

Discussion

In this article, the preparation of a new fluorescent analog of cholesterol 4 is reported along with some of its properties. By analogy with structurally similar synthetic glycolipids i.e. compound 5, the analog interacts much like cholesterol itself with phospholipid based membranes. Importantly, the molecule can be incorporated into membranes either directly or by an exchange route, making it a highly useful probe. Other fluorescent cholesterol analogs, such as *N*-(2-napthyl)-23,24-dinor-5-cholen-22-amin-3β-ol and the 3-pyrenemethyl ester of 5-cholenic acid-22,23-bisnor-3-β-ol have been reported in the literature [10,11]. These compounds contain rather drastic alterations in the cholesterol side chain in addition to being even more hydrophobic than cholesterol itself. No information has been reported as to whether these analogs can be incorporated into preformed membranes.

The impetus for the study of compound 4 was based on previous work done on synthetic cholesterol based glycolipids such as compound 5 [1–3]. These molecules were incorporated into membranes in much the same way as cholesterol itself, as determined by the various criteria mentioned earlier. Indeed, recent studies have shown that cholesterol ethers and esters will fully support growth in certain cholesterol auxotrophs [12]. Thus, attaching the fluorescent dye to the cholesterol oxygen was deemed a permissible substitution. NBD was chosen as the chromophore since it has suitable fluorescence properties as well as being a relatively simple molecule, especially for a usable fluorescent dye. In addition, an NBD containing cholesterol analog would be complementary to the NBD phospholipid analog currently under study in several laboratories to measure lateral phospholipid mobility [13,14]. The choice of the hydrophilic spacer group was dictated by two considerations. The water soluble nature of the spacer

decreases the likelihood that it will interact with the lipid domain and hence also decreases the likelihood that the NBD moiety will simply dissolve in the membrane. Furthermore, this hydrophilicity should increase the water solubility of the fluorescent analog as a whole over one containing a hydrophobic spacer, which makes it more probable that the molecule will exchange into membranes at a usable rate.

It has been demonstrated here that compound 4 can be incorporated into small unilamellar vesicles by soniating a mixture of the analog with phosphatidylcholine in buffer. These small unilamellar vesicles behaved quite similarly, by the criteria alluded to in the results section, to those prepared from phosphatidylcholine and the synthetic cholesterol based glycolipids. This is expected, since the critical cholesterol nucleus remains unmodified in both kinds of molecules.

In order that compound 4 be useful in cell biology there must be the possibility of exchanging it into preformed membranes. The incorporation of compound 4 into membranes was demonstrated in two ways. Incubation of non-fluorescent sonicated suspensions of compound 4 with phosphatidylcholine-based small unilamellar vesicles leads to the incorporation of compound 4 into the vesicles. Furthermore, phosphatidylcholine-based small unilamellar vesicles bearing compound 4 undergo exchange with underivatized small unilamellar vesicles. The rate of exchange is much slower than that for pure cholesterol under these conditions. This is to be expected since the rate of cholesterol analog exchange is thought to be determined, in a major way, by the solubility of the analog in water [9]. The fact that compound 4 exchanges with an appreciable rate at all is probably due to the presence of the hydrophilic spacer group.

The fluorescent analog discussed here can also be exchanged into living cells, such as Hela cells, both by transfer from sonicated suspensions containing compound 4 and by transfer from small unilamellar vesicles containing compound 4 (Rando, R.R. and Bangerter, F.W., unpublished data). Studies are under way to determine the membrane distribution and lateral mobility of compound 4 in living cells.

Acknowledgement

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