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Methyl-β-Cyclodextrins and Liposomes as Water-Soluble Carriers for Cholesterol Incorporation into Membranes and Its Evaluation by a Microenzymatic Fluorescence Assay and Membrane Fluidity-Sensitive Dyes

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A variety of methods to incorporate cholesterol into lipid membrane systems have been applied with varying success. We tested an incorporation method based on cholesterol-loaded methyl-β-cyclodextrins and compared it to a method that uses cholesterol-loaded liposomes. With methyl-β-cyclodextrin, we increased the cholesterol content in microsomal membranes to almost the fourfold of the original content. With cholesterol-loaded liposomes instead, we achieved an elevation of 140%. Short incubation times and well-defined carrier properties favor the β-cyclodextrin method. For direct detection of membrane cholesterol, we slightly modified a microenzymatic fluorescence assay originally developed for precise cholesterol detection in serum. Without the need to perform lipid extraction, this assay was reliable for cholesterol detection in liposomes and in microsomes. Additionally, we compared the sensitivity of the fluidity-sensitive fluorescent dyes pyrene, pyrene–methanol, bis-pyrene, 1-6-phenyl-1,3,5,-hexatrien, and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5,-hexatrien in order to detect cholesterol indirectly by the dynamically relevant changes exerted on lipid matrices. These dyes differ not only in their membrane location but also in their dynamical behavior. We calibrated the dyes in liposomes of defined cholesterol content and used the most suited ones to follow and quantify the cholesterol incorporation into liposomal and microsomal membranes.

Key Words: **methyl-β-cyclodextrins; cholesterol; membrane fluidity-sensitive dyes; fluorometric enzymatic cholesterol determination.**

As a major constituent of biological membranes, cholesterol plays a significant role for a great variety of physical membrane parameters and membrane-regulated processes (1). Thus, a well-defined regulation of membrane-bound cholesterol is essential for proper cell functioning. Artificial variation of the cholesterol content in membranes is used to learn about cholesterol-related processes in membranes and its possible effect on cell metabolism. Among the different enrichment procedures that are described in literature, two principally distinct methods can be discriminated.

One group of methods (in the case of whole cells) makes use of cell metabolism. In some cases, cholesterol content in cells is increased through diet *in vivo*, where standard animal food is mixed with 2–4% pure cholesterol (2, 3). In other cases, cholesterol content is changed in cell cultures or in freshly extracted cells *in vitro* (4, 5). Here, lipoproteins, which are characterized by their density as chylomicrones, very-low-density lipoprotein, LDL,[2] and HDL, are added to or withdrawn from buffer or serum. Lipoproteins consist of a monolayer of phospholipids and cholesterol, surrounding a core of triacylglycerins and esterified cholesterol molecules. Cellular receptors recognize ligands that are bound to the surfaces of the lipoproteins and regulate their endocytotic uptake through lysomal pathways. LDLs carry the maximum load of esterified cholesterol and are used to enrich cells with cholesterol. HDLs can diminish cellular cholesterol content probably by incor-

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[2] Abbreviations used: bis-pyrene, 1,3-bis-[1-pyrenyl]propane; Ch, cholesterol; DLPC, dilaurayl phosphatidylcholine; DPH, 1-6-phenyl-1,3,5,-hexatrien; E/M; excimer/monomer; HDL, high-density lipoprotein; LDL, low-density lipoprotein; *p*-HPA, *p*-hydroxyphenylacetic acid; Pl, phospholipid; TMA–DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5,-hexatrien.

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porating the molecules after diffusion through the aqueous phase (6), but collisions (7) and receptor-mediated mechanisms (8) have also been proposed (9). Since endogenous cholesterol synthesis in cells seems to be influenced by the extracellular content of HDL and LDL, a multiplicity of effects must be taken into account and must be carefully tested in every cell type of interest.

The other group of methods uses cholesterol-carrying complexes that normally are not involved in metabolic cell processes. Here, cholesterol exchange between the donor (carrier) and the target (membrane) follows partition equilibration. The free enthalpy difference ΔG between the carrier-bound cholesterol and the membrane-bound cholesterol determines the steady-state partition coefficient for cholesterol between these reservoirs. The transition rate into the steady-state equilibrium is additionally controlled by (i) the energy barrier to be overcome when cholesterol leaves the carrier, (ii) the diffusion of the poorly soluble molecule across the aquatic media, and (iii) the energy barrier to be overcome prior to incorporation into the target matrix.

Solubilization of hydrophobic molecules in aquatic media can be enhanced either by binding them covalently to hydrophilic complexes or by dissolving them in nonpolar suspensions. As hydrophilic carrier complexes, cholesterol-rich dispersions (10, 11), cholesterol-loaded liposomes (12), or cholesterol-coated Sephadex beads (13) have been applied. Among these methods, cholesterol-loaded liposomes is the most popular because of easy processing, but different incorporation results are reported (11, 12, 14, 15). Recently, cyclodextrins have been reported to mediate membranal cholesterol content very efficiently (16, 17).

Cyclodextrins enhance the solubility of hydrophobic substances and therefore should be considered as an effective carrier system. The cyclodextrin complexes are torus-shaped cyclic oligosaccharides consisting of 6, 7, or 8 (α, β, γ) glucopyranose units which confine a hydrophobic cave. Solubility of the naturally occurring cyclodextrins is increased by chemical substitution of the 2, 3, and 6 hydroxyl sites (18). The cavity size of the cyclodextrin complex seems to be the major determinant for the inclusion of a hydrophobic guest molecule. To obtain high incorporation rates of hydrophobic guest molecules into the cyclodextrin cavities, the temperature of a suspension containing both the cyclodextrins and the guest molecules must be decreased and the liquid must be extracted. In the case of cholesterol, Klein *et al.* (16) succeeded at incorporating 30 mg of cholesterol into 1 g of methyl-β-cyclodextrins by freeze-drying the suspensions. For our experiments, we used commercially available cholesterol-loaded methyl-β-cyclodextrins (42 mg/g) from Sigma (Deisenhofen). Loaded cyclodextrins release their single guest molecules when resuspended in liquids at temperatures around 37°C and via diffusion the molecules incorporate into undersaturated target matrices. It has already been shown that free β-cyclodextrins reliably and selectively deplete isolated or intact cellular membranes from cholesterol (9, 19) and that the cholesterol content can be reversibly restored or even increased in cells or membranes by cholesterol-loaded β-cyclodextrins (16, 20). In reasonable concentrations, cyclodextrins do not seem to provoke negative effects on cells (9, 20, personal observation in freshly extracted mice thymocytes). They not only find wide applications in *in vitro* cell cultures, but are also discussed for *in vivo* application (21).

In this study, we compare the cholesterol transfer from liposomes and from methyl-β-cyclodextrins into pig liver microsomes in order to obtain the method which offers better cholesterol incorporation rates.

For the quantification of cholesterol in membranes, a variety of methods like isotope dilution mass spectrometry, gas, thin-layer, and liquid chromatography, and colorimetric and fluorescence assays are used. In 1995, Grey *et al.* (22) showed that a microenzymatic fluorescence assay using *p*-HPA as a fluorophore yielded excellent results when applied to the detection of cholesterol in serum. Compared to standard colorimetric assays, sensitivity was improved by two orders of magnitude. Membrane-bound cholesterol can be shielded from the access of enzymatic assay compounds by the head groups of the membrane lipids (23). Among other substances, Triton X-100 is suggested to make cholesterol more accessible for the enzymes (24). Combined with a Triton X-100 treatment, this assay showed very good results in our hands when applied to liposomes and microsomes, but was troublesome when applied to whole cells.

To be sure that the directly measured cholesterol molecules in microsomes and liposomes had properly been incorporated into the membranes and have not formed micelles attached to the membranes, we developed an indirect method to follow varying cholesterol concentrations inside of membranes. It is known that membrane cholesterol content is an important determinant of membrane "fluidity" or "microviscosity." Thus, signals from fluidity-sensitive fluorescent dyes can also be interpreted in terms of indirect quantification of membrane cholesterol content. In 1988, MacDonald *et al.* (25) observed a linear decrease of the fluidity signals from pyrene and DPH in liposomes and in protein-rich microsomal vesicles with decreasing temperature, increasing pressure, and (only in liposomes) increasing cholesterol contents. According to Cheng *et al.* (26), cholesterol exerts differential effects not only on the conformational dynamics of acyl chains at different depths of the membrane but also on the

rotational and translational dynamics of the acyl chains.

To find the most sensitive dye for the detection of cholesterol-induced changes of membrane dynamics, we compared the fluorescent dyes pyrene, pyrene–methanol, bis-pyrene, DPH, and TMA–DPH, which differ in their membrane locations and their dynamic behavior (27).

For this study, we calibrated these dyes with liposomes of defined cholesterol content and used the most suited ones to indirectly quantify the cholesterol incorporation from β-cyclodextrins into liposomal and microsomal membranes.

MATERIALS AND METHODS

Liposome preparation. Egg phosphatidylcholine (Sigma, Deisenhofen, Germany, 99.3% Pl) and varying amounts of pure cholesterol (Sigma) were dissolved in 5 ml dichlormethane–methanol (2:1) to a final concentration of 1×10^{-2} M. In a vacuum rotary evaporator liquid was removed at 30°C until a lipid film smoothly covered the glass tube walls. After overnight storage in vacuum at 50°C (extraction of residual solvent), the lipid film was resuspended in Tris buffer solution (100 mM Tris, pH 7.4) by vortexing to give a concentration of about 2 mg/ml. At 50°C samples were sonicated for 15 min followed by ultracentrifugation (105,000*g*, 1 h) to dispose unincorporated lipids in the pellet. Final lipid concentrations were determined according to Stewart (28). Contact to oxidizing atmosphere was avoided at all steps by flushing with nitrogen. Preparations were stored under nitrogen in darkness at 4°C and used within days.

Microsome preparation. Microsomal fractions from freshly obtained pig livers (public slaughterhouse) were prepared by differential centrifugation according to Lu (29). Microsomes were suspended in 10 mM potassium phosphate buffer, pH 7.4, and stored at −80°C. Microsomal protein content (29.22 ± 0.44 mg protein/ml suspension) was determined according to Peterson (30). Microsomal phospholipid content (assumed average molecular weight 750 g/mol) was estimated according to Kaschny (31) [protein/Pl = 2:1 (w/w)]. Microsomal cholesterol (see the following description of cholesterol determination) content was determined to be 6.1 ± 0.3 mol% (mol cholesterol/mol phospholipids · 100).

Direct cholesterol determination. For cholesterol quantification in solutions, in liposomes, and in microsomes, a microenzymatic fluorescence assay for serum cholesterol (22) was slightly modified. The fluorescence assay mixture contained 0.6 U/ml cholesterol esterase, 0.6 U/ml cholesterol oxidase, 6.0 U/ml peroxidase (horseradish), 3 mM *p*-HPA, and 10 mM sodium cholate dissolved in phosphate buffer (0.1 M, pH 7.4). All reagents were purchased from Sigma and used without further purification. Only deionized water was used. Calibration solutions contained pure cholesterol in ethanol. Stored at −20°C, the assay mixture could be used for several months. For quantifying procedures, 100 μl assay mixture and 10 μl of 10% Triton X-100 were added to sample volumes of 5 to 20 μl. Phosphate buffer was added to a final assay volume of 200 μl. As standards, pure ethanol or cholesterol-free liposomes were used in corresponding buffers. After vortexing for 10 s, free cholesterol solutions were incubated for 30 min, but liposomes and microsomes for 8 h, to guarantee total cholesterol oxidation. After incubation, 1.8 ml of distilled water was added to the samples and the fluorescence intensity of *p*-dihydroxyphenylacetic acid was monitored at 406 nm under excitation at 320 nm with a Kontron SFM 25 spectrofluorometer (Kontron, Eching, Germany) equipped with an L-format anisotropy inset and a Julabo HC thermostat (±0.5°C) (Julabo Labortechnik, Seelbach, Germany). Calibration curves, obtained from linear regression analysis, proved the test to be linear over three orders of magnitude (1×10^{-10} to 1×10^{-7} mol/ml). Free cholesterol in solution was determined with an accuracy of less than 2.5%. Membrane-bound cholesterol was detected with an error of less than 10%.

Dye-incubation and fluidity measurements. The fluidity-sensitive fluorescent dyes pyrene, pyrene–methanol, and bis-pyrene (Molecular Probes, Eugene, OR) were dissolved in ethanol (Merck, Darmstadt, Germany). DPH and TMA–DPH were dissolved in dimethylformamide (Janssen, Geel, Belgium). Equivalents from stock solutions were added to 2 ml of liposomal or microsomal suspensions, containing 0.1 mg/ml [lipid in buffer], so that a molar ratio ([lipid]:[dye]) of 100:2 was obtained in the case of pyrene and pyrene–methanol, of 10,000:1 in the case of bis-pyrene (to neglect intermolecular excimer formation), and of 400:1 in the case of DPH and TMA–DPH. Light-protected incubation at room temperature was carried out until E/M or fluorescence anisotropy signals were stable (maximum 1 h in the case of TMA–DPH). All measurements were carried out in quartz cuvettes of 1 cm^2 cross-section with the above-mentioned spectrofluorometer. For calculations of the diffusion-controlled E/M ratio of pyrene and pyrene–methanol and for the E/M ratio of bis-pyrene, which represents intramolecular dynamical and configurational dependencies (32), dyes were excited at 336 nm. Emission intensities were recorded at 395 nm for monomer emission and at 475 nm for excimer emission. E/M values from pyrene and pyrene–methanol were interpreted as fluidity (or inverse viscosity) parameters of the membranes according to

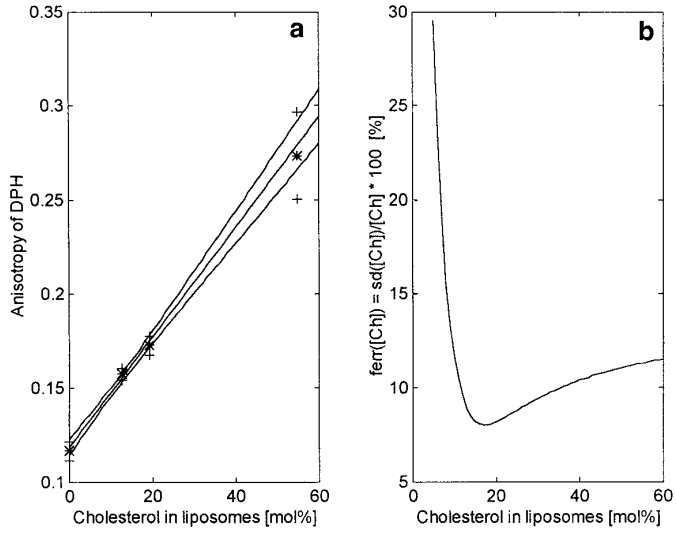

FIG. 1. Linear regression analysis of DPH anisotropy in liposomes of defined cholesterol content. (a) Means (*) of four to five liposomal samples with corresponding standard deviations (+) and linear regression line (r_{DPH} = 0.999) with its (standard deviation) error margins. (b) The error function $f_{err}([Ch])$ = SD([Ch])/[Ch] · 100, calculated from linear regression data of the DPH anisotropy values, is shown. It possesses a minimum error of about 8% (1.44 mol%) at a liposomal cholesterol content of about 18 mol%.

Birks (33). For bis-pyrene, E/M interpretation followed Cheng *et al.* (26).

For DPH and TMA–DPH, the fluorescence anisotropy was calculated according to Lakowicz (34):

$$r = \frac{I_{II} - G \cdot I_{\perp}}{I_{II} + 2 \cdot G \cdot I_{\perp}},$$

where r is the steady-state fluorescence anisotropy, I_{II} and $I_{\perp}$ are the fluorescence intensities at 425 nm parallel and perpendicular to the polarization of the excitation light at 358 nm, and G is the "grating factor."

To characterize the suitability of the fluorescent dyes for the detection of cholesterol-induced fluidity changes in lipid bilayers, two parameters were used: "sensitivity" and "min(f_{err}([Ch]))." The ratio of the maximum and the minimum of the fluorescence signals of the dyes at certain cholesterol contents was interpreted as sensitivity. An error function $f_{err}([Ch])$ = SD([Ch])/[Ch]) · 100 was calculated and is shown in the case of DPH anisotropy in Fig. 1b. SD([Ch]) represents the standard deviation of the linear regression line as a function of a given cholesterol content in liposomes. The DPH anisotropy values, their linear regression line, and the corresponding standard deviation error margins are shown in Fig. 1a. By definition, the error function $f_{err}([Ch])$ indicates how much a given cholesterol content must be changed in order to be detected by the corresponding dye. The minimum of this function [min(f_{err}([Ch]))] indicates the smallest possible percentile change detectable in liposomes containing the indicated cholesterol content.

Cholesterol incorporation into microsomes by cholesterol-loaded liposomes. The highest possible amount of cholesterol that we could incorporate into liposomes during the preparation procedure resulted in a molar ratio of 1:1 (Ch:Pl). Additional cholesterol was removed during ultracentrifugation. Various amounts of liposomal suspensions (as indicated in Fig. 3) were added to 5-ml aliquots of microsomes and incubated at 37°C for 2 h under gentle stirring. Afterward, the microsomes were separated from liposomes by ultracentrifugation (2 h at 105,000*g*). The microsomal pellet was resuspended in potassium phosphate buffer to its original volume and the protein content was determined with Peterson's method to control possible losses of microsomal material during the procedure.

Cholesterol incorporation into microsomes by cholesterol-loaded β-cyclodextrins. Various amounts of cholesterol-containing methyl-β-cyclodextrins (Sigma, Deisenhofen, 42 mg Ch/g methyl-β-cyclodextrin) were added to 5-ml aliquots of microsomal suspensions and incubated at 37°C for 1/2 h under gentle stirring. Afterward, microsomes were separated from β-cyclodextrins by ultracentrifugation (2 h at 105,000*g*). The microsomal pellet was resuspended in potassium phosphate buffer to the original volume. Protein content was determined as before. As a control, microsome free potassium phosphate buffer was mixed with the maximum amount of β-cyclodextrins as indicated in Fig. 3 to affirm that no β-cyclodextrins precipitated during ultracentrifugation.

For both preparation methods resuspended microsomes were centrifuged a second time to affirm that the supernatant was cholesterol free.

Membrane fluidity measurements in microsomes and liposomes during cholesterol enrichment with β-cyclodextrins. Cholesterol-free liposomes or microsomes were suspended in Tris buffer solution (100 mM Tris, pH 7.4, 0.1 mg Pl/ml buffer) and incubated with pyrene, bis-pyrene, or DPH as described above. Then, 2 or 10 times the original amount of cholesterol in microsomes was added to the dye-labeled microsomal suspensions using methyl-β-cyclodextrins as cholesterol carriers. In the case of liposomes, cholesterol in β-cyclodextrins was added to give a final concentration of 1:1 or 2:1 (Ch:Pl). Under gentle stirring, probes and controls were heated to 40°C for 1/2 h in order to optimize the diffusion-controlled flux of single cholesterol molecules from the hydrophobic cavities of the β-cyclodextrins to the thermodynamically undersaturated acceptor membranes of the microsomes or the liposomes (18). After cooling

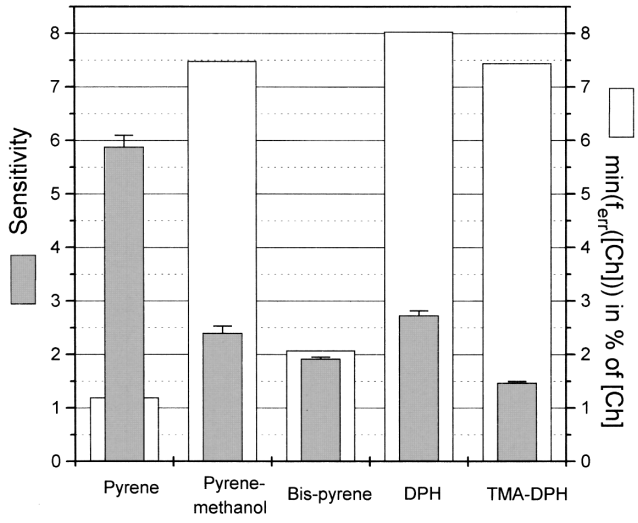

FIG. 2. Sensitivity (as defined under Materials and Methods) and minimum of the error function f_{err}([Ch]) for the detection of liposomal cholesterol content by means of fluidity-sensitive fluorescent molecular labels. Membrane dynamics of four to five liposomal preparations with defined cholesterol content ranging from 0 to 50 mol% [Ch/(Ch + Pl) · 100] were determined for the indicated membrane dyes. In all cases the different fluidity signals (intermolecular E/M ratio of pyrene and pyrene–methanol, intramolecular E/M ratio of bis-pyrene, and the anisotropy of DPH and TMA–DPH) yield good linear correlations with the cholesterol content in liposomes. Sensitivity values are plotted as gray shaded columns. White columns represent the minimum of the corresponding error functions.

the probes to 25°C, the fluidity signals were determined as described before.

All measurements were carried out in quartz cuvettes of 1 cm^2 cross-section with the above-mentioned Kontron SFM 25 spectrofluorometer.

RESULTS AND DISCUSSION

Membrane fluidity-based cholesterol detection using various fluorophores. The calibrations with different fluidity-sensitive membrane dyes in liposomes of defined cholesterol concentrations are shown in Fig. 2. In general, the fluidity signals of the fluorophores yield excellent linear responses in correlation to increasing cholesterol contents (see Fig. 1 for DPH). These findings are in accordance with MacDonald *et al.* (25) who measured DPH and pyrene signals in cholesterol-containing DLPC liposomes. In membranes consisting of either pure DLPC and dimyristoyl PC, or of mixed DLPC/dipalmitoyl PC, Parasassi *et al.* (35) detected the formation of particularly ordered molecular microdomains at several critical cholesterol concentrations which they evaluated with the "generalized polarization" term of the membrane fluorophore Laurdan. In egg yolk liposomes, our dyes did not reveal any particular microdomain structures.

With a linear regression coefficient of $r_p = 0.989$, pyrene possesses the highest sensitivity and the best error function for the detection of cholesterol in liposomal membranes. Pyrene E/M signals from cholesterol-free liposomes are six times higher than from cholesterol-saturated liposomes. The error function (see Materials and Methods) of the pyrene calibration line drops down to almost 1% at the point of best detection. The intramolecular excimer kinetics of bis-pyrene is less sensitive toward cholesterol-induced variations of the membrane fluidity than pyrene, but due to good linear responses of the E/M signal and good reproduction of the values (r_{bp} = 0.993) the bis-pyrene error function possesses a minimum of about 2%. The sensitivity of the DPH (r_{dph} = 0.999) anisotropy is somewhat higher than that of bis-pyrene, but due to higher scattering of the anisotropy values, a more unfavorable error function is obtained. The E/M signals of pyrene–methanol (r_{pm} = 0.994) as well as the TMA–DPH anisotropy values (r_{tma} = 0.982) show less sensitivity combined with rather moderate error functions.

Pyrene and DPH values show more sensitivity toward cholesterol variations in liposomes than their derivatives. Due to their hydrophilic appendices, the movement of the derivatives is restricted to an area near the head group region of the bilayers (27). While DPH anisotropy values range from about 0.12 in cholesterol-free liposomes to about 0.3 in cholesterol-saturated liposomes, TMA–DPH anisotropy values range only from 0.17 to 0.25. This confirms that the head group region of pure lipid matrices is more rigid than its core. With increasing cholesterol content instead, DPH is left with less rotational freedom than TMA–DPH. E/M values of pyrene start at 0.46 in cholesterol-free liposomes and fall to 0.08 in cholesterol-saturated liposomes. Pyrene–methanol values instead range from 0.18 to 0.08. Diffusion of the pyrenes in the core of the cholesterol-free lipid matrix is fast compared to the diffusion processes near the head groups. Nevertheless, diffusion rates in the core and near the surface approach each other in the cholesterol-saturated matrix suggesting in this case comparable rigidity in both regions.

Although linear regression analysis favors pyrene to monitor fluidity changes in lipid membranes induced by cholesterol, one should keep in mind that the intermolecular excimer kinetics of pyrene is a process controlled not only by the rate of diffusion, but also by the pyrene content in the lipid matrix. If the partition coefficient between matrix and media is changed, the E/M value of the dye will also change, although the diffusion coefficient might not have changed inside of the matrix. The same holds for pyrene–methanol. If intermolecular excimer formation kinetics is ruled out by low dye concentrations inside the membrane, the intramolecular E/M signal of bis-pyrene is a reliable

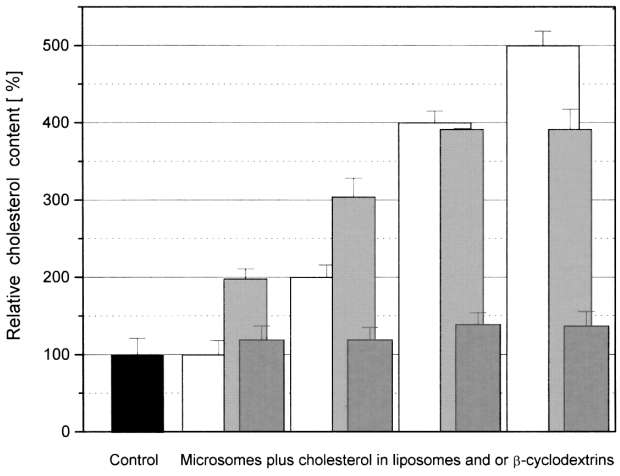

FIG. 3. Microsomal cholesterol content before and after the incubation procedure with cholesterol-loaded liposomes or methyl-β-cyclodextrins. Natural cholesterol content in microsomes was determined (6.1 ± 0.3 mol%) and set to 100% for the control (black column). White columns: added amounts of cholesterol in liposomes or in methyl-β-cyclodextrins are given relative to the microsomal cholesterol content of the control microsomes. After the incubation procedures (see Materials and Methods), resulting cholesterol content in the microsomes is shown in dark gray columns for incubation with liposomes and in light gray columns for incubation with methyl-β-cyclodextrins. For both methods saturation levels for the incorporation of cholesterol into microsomal membranes are achieved when more than fourfold the original cholesterol content is added, but β-cyclodextrins show much better incorporation rates.

kinetic parameter, independent of fluctuations of the partition coefficient between media and membrane. For TMA–DPH and DPH, small changes of the partition coefficient do not affect anisotropy signals to a high degree.

Cholesterol incorporation. Cholesterol incorporation into microsomal membranes after incubation with cholesterol-loaded β-cyclodextrins or liposomes is shown in Fig. 3. The columns show that while adding up to the double amount of cholesterol in β-cyclodextrins, virtually all cholesterol molecules are incorporated into the microsomal membranes, while liposomes with the same amount of cholesterol cause a much smaller net flux of cholesterol. When incubating with a fourfold amount of cholesterol in β-cyclodextrins or in liposomes, cholesterol saturation of the microsomal membrane is reached and further addition of cholesterol has no effect. With both carriers, cholesterol incorporation is well reproducible. The application of the β-cyclodextrin complex is favored because of short incubation times (1/2 h in the case of β-cyclodextrins and 2 h in the case of liposomes), well-defined carrier properties, and its efficiency. When working with β-cyclodextrins, side effects can easily be controlled by incubating cells or membrane suspensions with unloaded β-cyclodextrins. Cholesterol-carrying liposomes instead may transfer lipids to microsomal or cellular membranes or may even cause membrane fusions.

To monitor relative fluidity changes in microsomal and liposomal membranes induced by a net flux of cholesterol from β-cyclodextrins (Figs. 4 and 5), we used the fluorescent dyes pyrene, DPH, and bis-pyrene because their individual fluidity signals showed good sensitivity and error functions in our calibration measurements (Figs. 1 and 2). After incubation with twice the original microsomal cholesterol content of 6.1 ± 0.3 mol%, the microsomal fluidity signals decreased 10% applying pyrene, 9% applying bis-pyrene, and 13% applying DPH (Fig. 4). When incubated with a 10-fold concentration of natural cholesterol, signals dropped to 74% with pyrene and to 79% with bis-pyrene. The corresponding molar cholesterol content inside the membranes was also calculated from linear regression analysis. We found a mean rise of 9.8 ± 2.4 mol% (Ch/Pl) which means that with this method 81 ± 19% of the added cholesterol was actually translocated into the microsomal membrane during incubation with 2-fold of its original cholesterol content. A mean value of 23.4 ± 5.9 mol% (obtained with pyrene and bis-pyrene) was calculated in the case of incubation with the 10-fold amount of natural cholesterol. Here the results from the direct measurements of the cholesterol

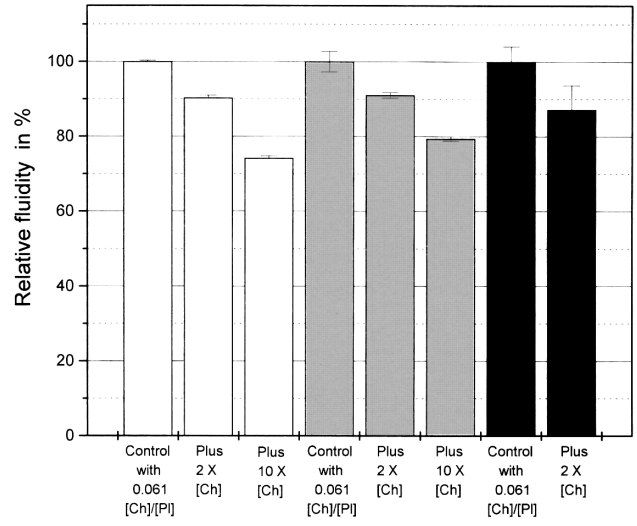

FIG. 4. Cholesterol-induced fluidity changes in microsomal membranes as detected by pyrene (white columns), bis-pyrene (gray columns), and DPH (black columns). 100% is set for the fluidity values of untreated microsomal membranes containing 6.1 ± 0.3 mol% of cholesterol with respect to their phospholipid content. The fluidity of microsomal membranes incubated with double (2×) their original cholesterol content was determined by the diffusion-controlled intermolecular excimer method with pyrene, the intramolecular excimer method with bis-pyrene, and the anisotropy method with DPH. The fluidity changes of microsomes incubated with 10-fold (10×) the original cholesterol content was additionally determined with pyrene and bis-pyrene. Results shown are means of three to four independent experiments with corresponding standard deviations.

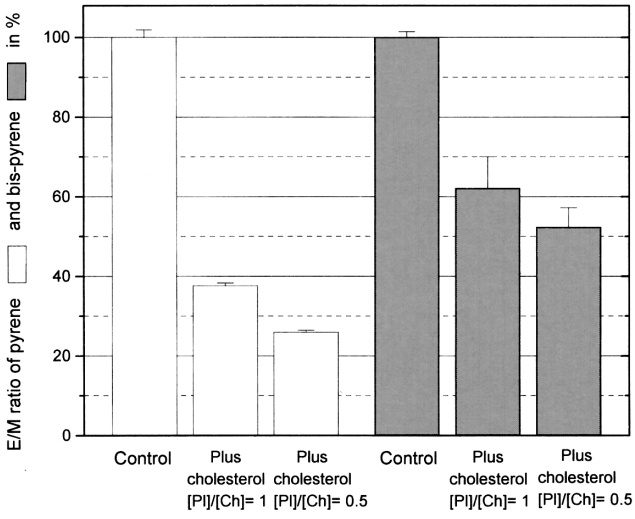

FIG. 5. Cholesterol-induced fluidity changes in liposomal membranes as detected by pyrene (white columns) and bis-pyrene (gray columns). 100% is set for the fluidity values of cholesterol-free liposomes. The fluidity changes of liposomal membranes incubated with a cholesterol content equal to their phospholipid content (Pl/Ch = 1) and with double their phospholipid content (Pl/Ch = 0.5) were determined by the diffusion-controlled intermolecular excimer method with pyrene and the intramolecular excimer method with bis-pyrene. Results shown are means of three to four independent experiments with corresponding standard deviations.

incorporated into the microsomal membranes with the *p*-HPA enzymatic assay (Fig. 3) and the fluorophore-dependent fluidity method are in agreement within their error margins.

The fluidity signals of pyrene and bis-pyrene also decreased drastically in liposomes when incubating them with cholesterol in β-cyclodextrins yielding molar ratios of Pl:Ch = 1:1 and Pl:Ch = 1:2 (Fig. 5). Compared to cholesterol-free liposomes, the pyrene fluidity signals drop to 38% in the first case and to 26% in the latter. The bis-pyrene signals drop to 62 and 52%, respectively. These values correspond to cholesterol incubations of 43.3 $\pm$ 4.3 and 51.2 $\pm$ 14.5 mol% for pyrene and to 55.1 $\pm$ 3.5 and 68.1 $\pm$ 4.4 mol% for bis-pyrene, respectively. In this case, the pyrene signals seem to give more reliable information since they detect an incorporation efficiency of 87% when cholesterol is added at a molar ratio of 1 (Pl/Ch) and report almost exactly the expected saturation molar ratio of 1:1 (Pl:Ch) in liposomes when cholesterol is added at a molar ratio of 0.5 (Pl/Ch).

The results from the direct cholesterol detection in microsomes (Fig. 3) clearly favor β-cyclodextrins and not the conventional method with liposomes. From the indirect cholesterol detection in microsomes (Fig. 4) and in liposomes (Fig. 5), we find that β-cyclodextrins incorporate cholesterol into the target matrices in a very fast, reproductive, and efficient manner until saturation of the membranes is reached. Their well-defined carrier properties, their suitability for cell cultures, and the easy handling during the incubation procedures recommend them as a powerful tool for investigations of cholesterol-related effects in cellular and artificial membranes.

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