Use of cyclodextrins for manipulating cellular cholesterol content

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Abstract Previous studies from this laboratory have demonstrated that exposure of tissue culture cells to cyclodextrins results in rapid cholesterol depletion. In the present study, we have developed experimental systems for using solutions of cyclodextrins, either 2-hydroxypropyl β-cyclodextrin or methylated β-cyclodextrin, complexed with varying amounts of free cholesterol to manipulate cell cholesterol content. Cholesterol delivered via the cyclodextrin has been found to be metabolically active, as measured by the acyl-coenzyme A:cholesterol acyltransferase (ACAT)-mediated esterification of [^{3}H]cholesterol in Fu5AH rat hepatoma cells and Chinese hamster ovary cells. The methylated β-cyclodextrin was found to be a more efficient donor in all cell types studied, with an average cholesterol uptake of at least 100 μg cholesterol/mg protein within 6 h. By modifying the cyclodextrin:cholesterol molar ratio, it is possible to manipulate the cellular cholesterol content of cells, producing conditions ranging from net cholesterol enrichment to depletion. The use of cyclodextrins provides a convenient, precise and reproducible method for modulating the cholesterol content of tissue culture cells.—**Christian, A. E., M. P. Haynes, M. C. Phillips, and G. H. Rothblat.** Use of cyclodextrins for manipulating cellular cholesterol content. *J. Lipid Res.* 1997. **38:** 2264–2272.

Supplementary key words cholesterol • cyclodextrins • tissue culture • cholesteryl ester

β-Cyclodextrins (β-CD) are cyclic oligosaccharides consisting of 7-β (1–4)-glucopyranose units. The external faces of the cyclodextrin molecule are hydrophilic, whereas the internal cavity is lined with C(3)H and C(5)H hydrogens and ether-like oxygens that provide an hydrophobic environment. This internal cavity has the ability to encapsulate normally hydrophobic, thus insoluble, compounds and allow them to become soluble in aqueous solutions (1, 2). Cyclodextrins (CD) have been used extensively as drug delivery vehicles (1, 3) and chemically modified β-CDs, such as 2-hydroxypropyl β-cyclodextrins (2OHpβCD) and methylated β-cyclodextrins (MβCD), have been made that can affect characteristics such as solubility, complex formation, and toxicity (4–6). In vitro, β-CDs have a high affinity for sterols as compared to other lipids (6, 7), which may make these compounds quite effective in modifying cholesterol metabolism in vivo (6, 8).

Recently, a series of studies have demonstrated that cyclodextrins are very efficient in stimulating the removal of cholesterol from a variety of cells in culture (9–11). The exposure of cells to high concentrations of β-CDs, especially modified β-CDs (10–100 mM) results in rates of cell cholesterol efflux far in excess of those achieved with physiological cholesterol acceptors such as HDL. This rapid efflux has been used to demonstrate the presence of different kinetic pools of cholesterol within cells. At lower concentrations (≈1 mM), β-cyclodextrins have been shown to function as cholesterol shuttles that can catalyze the exchange of cholesterol between cells and serum lipoproteins (12). In addition, cyclodextrins have been used recently to monitor movement of cholesterol from monolayers (13) and the intracellular movement of cholesterol in tissue culture cells (14).

The mechanism that allows cyclodextrins to remove cholesterol from cell membranes so efficiently is related to their ability to reduce the activation energy for cholesterol efflux from the approximately 20 kcal/mol required for movement of cell cholesterol to phospholipid acceptors to a value of 7–9 kcal/mol (10). This difference has been attributed to the need for cholesterol molecules in the plasma membrane to desorb completely into the aqueous phase before being absorbed by HDL particles or phospholipid liposomes, whereas the membrane cholesterol molecules can incorporate directly into the hydrophobic cavity of the

Abbreviations: ACAT, acyl-coenzyme A:cholesterol acyltransferase; β-CD, beta-cyclodextrin; BSA, bovine serum albumin; CHO, Chinese hamster ovary cells; dH_2O, deionized water; DMEM, Dulbecco's minimum essential medium; EMEM, Eagle's minimum essential medium; FBS, fetal bovine serum; GLC, gas–liquid chromatography; HDL, high density lipoprotein; 2OHpβCD, 2-hydroxypropyl β-cyclodextrin; MβCD, methylated β-cyclodextrin; MPM, mouse peritoneal macrophages; PBS, phosphate-buffered saline.

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cyclodextrin molecule without the necessity of traveling through any intermediate aqueous phase (10, 15). It is this ability to directly transfer cholesterol between cell membranes and cyclodextrins that enables them to be efficient donors as well as acceptors of cholesterol. Several researchers have used cyclodextrins complexed with cholesterol and other sterols as a method to enrich cellular cholesterol content (16–21). However, the methodology for using cyclodextrin:cholesterol complexes as cholesterol delivery vehicles in cell culture systems has not been investigated in detail, and no standardized procedures have been suggested. As cyclodextrins have the potential for being valuable tools for experimentally manipulating cell sterol content or composition, we initiated the present study to establish well-controlled conditions for preparing cyclodextrin:cholesterol complexes and modifying cell cholesterol content. In addition, we have performed experiments to demonstrate that cholesterol delivered to cells by cyclodextrins becomes metabolically available in several cell types, as demonstrated by the esterification of the cholesterol by acyl-coenzyme A:cholesterol acyltransferase (ACAT).

MATERIALS AND METHODS

Materials

2OHpβCD was obtained from Cyclodextrin Technologies Development, Inc. (CTD) (Gainesville, FL) or as a generous gift from Cerestar USA, Inc. (Hammond, IN). MβCD was also kindly provided by Cerestar USA, Inc. [1,2-^{3}H]cholesterol (51 Ci/mmol in EtOH) was purchased from DuPont NEN (Boston, MA) and [^{14}C]2OHpβCD (6.95 mCi/g in dH_2O) was a generous gift from CTD. Eagles minimal essential medium (EMEM), Dulbecco's minimal essential medium (DMEM) and Hams F12 media were obtained from BIO-Whittaker. Calf serum, heat-inactivated fetal bovine serum (FBS), gentamicin, and cholesterol were purchased from Sigma (St. Louis, MO). Pfizer ACAT inhibitor, CP-113, 818 was a gift from Pfizer Pharmaceuticals (Groton, CT).

Preparation of cyclodextrin:cholesterol complexes

The following procedure was used to prepare 10 mL of a 2.5 mM MβCD:cholesterol solution with a CD:cholesterol molar ratio of 10:1 (sp act = 12.5 μCi/mg [^{3}H]cholesterol). The protocol may be adapted for a wide concentration range of MβCD or 2OHpβCD or molar ratio of cyclodextrin:cholesterol (Table 1). A volume of 16 μL of cholesterol from a stock solution at 50 mg/mL, in chloroform:methanol 1:1 (v:v), was added to a glass tube. The appropriate amount of radiolabeled cholesterol was then added to produce the desired specific activity. The solvent was evaporated under a gentle stream of nitrogen. A mass of 33.45 mg of MβCD (average mol wt 1338, lot no. E 8039, Cerestar USA, Inc.), which had been dissolved in 10 mL of aqueous solution, was then added. The tube was vortexed to bring the dried cholesterol off the wall of the tube and then sonicated in bath sonicator for 1–3 min. This 100% saturated cyclodextrin:cholesterol solution was incubated in a rotating water bath at 37°C overnight. Immediately before using the solution, it was filtered through a 0.45 μm syringe filter (Millipore, Bedford, MA) to remove excess cholesterol crystals. There was no loss of either 2OHpβCD or MβCD upon filtration as determined by the recovery of [^{14}C]2OHβpCD or by a colorimetric charring assay for the MβCD. The cyclodextrin:cholesterol solutions used in the cell experiments were prepared by the direct addition of cholesterol to the cyclodextrin in the appropriate molar ratio as described above.

Determination of cyclodextrin:cholesterol molar ratios

A 100 mM solution of 2OHpβCD was saturated with cholesterol labeled with [^{3}H]cholesterol (sp act = 2.8 × 10^8–1.3 × 10^{10} cpm/mmol) by incubating crystalline cholesterol with the cyclodextrin in a glass tube, as described above, followed by filtration to remove the excess cholesterol. This procedure yielded a cyclodextrin:cholesterol molar ratio of approximately 20:1. The 100 mM stock solution, maintained at room temperature, was then serially diluted by the addition of deionized H_2O. Aliquots of the individual dilutions were filtered to remove precipitated cholesterol and taken for liquid scintillation counting in a Beckman LS1801 liquid scintillation counter (Irvine, CA). The concentration of the cholesterol in the filtrate was quantitated based on the specific activity of the original cholesterol added to the 100 mM stock solution.

In contrast to the response observed with 2OHpβCD in which dilution of a cyclodextrin complex saturated with cholesterol resulted in the release of excess cholesterol from the complex, the dilution of a solution of MβCD saturated with cholesterol and labeled with [^{3}H]cholesterol (sp act = 8.9 × 10^7–8.9 × 10^8 cpm/mmol) appeared to enhance the capacity of the MβCD to complex cholesterol. Thus, to determine the saturation limits of various concentrations of MβCD, individual solutions at the indicated concentrations of MβCD were incubated with excess cholesterol labeled with [^{3}H]cholesterol (sp act = 8.9 × 10^7–8.9 × 10^8 cpm/mmol) for 24 h, followed by the removal of unsolubilized cholesterol by filtration, as described above. It is important to note that variation between lot numbers of

cyclodextrins was seen when determining cyclodextrin:cholesterol molar ratios. However, the difference in these molar ratios did not affect the loading capabilities of the cyclodextrin:cholesterol complexes.

Preparation of cyclodextrin:cholesterol complexes at varying molar ratios

For the purpose of these experiments, a cyclodextrin solution was considered to be 100% saturated when it solubilized the maximum amount of cholesterol, as described above. Preparations of cyclodextrins containing lower levels of cholesterol were used in a number of experiments. The concentration of cholesterol in these preparations was designated in terms of the saturation ratio of the complex. Thus, a '50% saturated' solution contained one-half of the amount of cholesterol needed to achieve saturation. Actual cyclodextrin:cholesterol molar ratios are shown in the tables and figures.

Cell culture

Media were buffered with sodium bicarbonate and cells were cultured in a humidified incubator at 37°C with 5% CO_2. All media were supplemented with 50 μg of gentamicin/mL. Fu5AH rat hepatoma cells were cultured in EMEM supplemented with 5% calf serum. Chinese hamster ovary (CHO) cells were cultured in Hams F12 media supplemented with 7.5% FBS. Elicited mouse peritoneal macrophages and GM3648A normal human skin fibroblasts were cultured in EMEM with 10% FBS.

Modification of cellular cholesterol using cyclodextrin:cholesterol complexes

Cells were plated in 36-mm wells 2–4 days prior to the experiments (2.2×10^6 cells per well). When the cell monolayers were approximately 90% confluent, the monolayers were washed 3× with prewarmed EMEM (37°C) after which the cyclodextrin:cholesterol solution was added (1 mL/well). The composition of the incubation media (cyclodextrin type, molar ratio, and cholesterol concentration) is indicated in the tables and figures. At the end of the incubation period, the medium was removed and the cell monolayer was washed 3× with cold PBS (4°C). Lipid was extracted from washed cell monolayer using isopropanol as previously described (22), and the amount of [^{3}H]cholesterol incorporated into cells was determined by liquid scintillation counting. The distribution of the labeled cholesterol between free and esterified cholesterol was determined after thin-layer chromatography (23). Total and free cholesterol mass analysis was done by gas–liquid chromatography (GLC) as previously described (24, 25). Cell protein was determined on the lipid-extracted monolayer using a modification (26) of the method of Lowry et al. (27). The quantitation of the incorporation of total [^{3}H]cholesterol mass from the cyclodextrin:cholesterol complexes was calculated based on the specific activity of the cholesterol at the time the cyclodextrin:cholesterol complexes were added to the cells. These values were compared to the actual mass of cell cholesterol as determined by GLC analysis. The distribution of cholesterol between cellular pools of free and esterified cholesterol was established on both the basis of direct mass measurements (GLC) and the distribution of radiolabeled cholesterol obtained through thin-layer chromatography.

Colorimetric charring assay for the quantitation of MβCD

Quantitation of MβCD in solution was determined by a colorimetric assay. A 10 mM solution of MβCD was prepared in deionized H_2O. A serial dilution was performed as previously described. A 1 mL aliquot of each sample was filtered through a washed 0.45-μm syringe filter. One-half milliliter H_2SO_4 (100%) was added to each sample and then heated at 100°C for 1 h. The absorbance at 320 nm of the resulting product, which was linearly correlated to MβCD concentration, was determined in a Beckman DU-40 spectrophotometer.

RESULTS

Characteristics of cyclodextrin:cholesterol complexes

The solubilization of cholesterol with varying concentrations of 2OHpβCD and MβCD is shown in **Fig. 1.** The cyclodextrin:cholesterol solutions were prepared in deionized H_2O as described in Materials and Methods. Upon dilution from 100 mM to 10 mM, the [^{3}H]cholesterol precipitated out of the [^{14}C]2OHpβCD:[^{3}H]cholesterol solution which caused an increase in the 2OHpβCD:cholesterol molar ratio from about 20:1 to 70:1 (Fig. 1A). In contrast, serial dilution of the MβCD:[^{3}H]cholesterol solution from 10 mM to 2.5 mM did not affect the MβCD:[^{3}H]cholesterol molar ratio. However, an increase in the molar ratio was observed when the concentration of the MβCD was below 2.5 mM. The reason for the differences in the complexation behavior of the two cyclodextrins are currently under investigation.

In the course of these studies we investigated the stabilities of the cyclodextrin:cholesterol complexes upon storage. [^{14}C]2OHpβCD:[^{3}H]cholesterol and MβCD:[^{3}H]cholesterol solutions were prepared as described in Materials and Methods. The recovery of both the cyclodextrin and cholesterol was monitored over a 6-

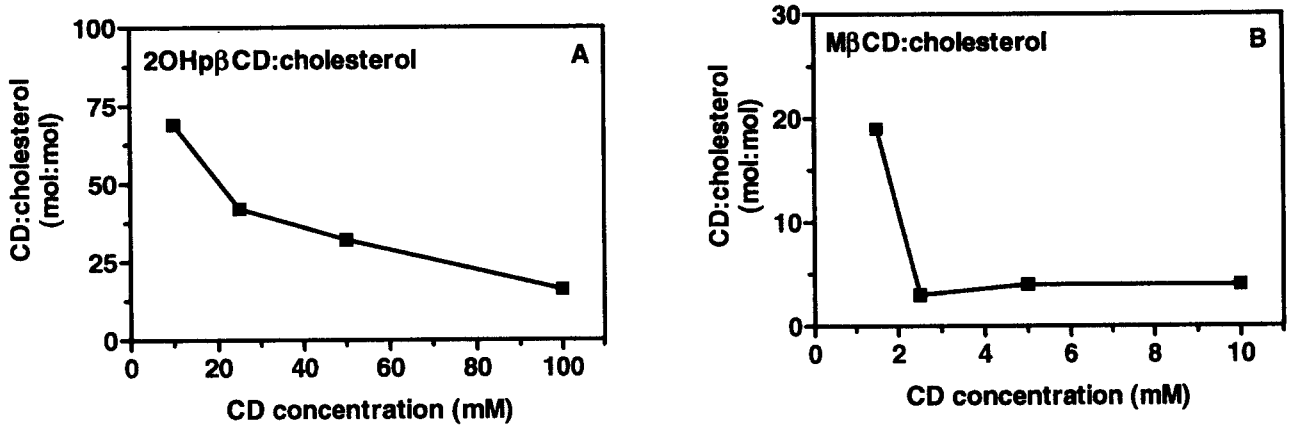

Fig. 1. Determination of cyclodextrin:cholesterol molar ratios. [^{3}H]cholesterol was complexed with either 2OHpβCD (100 mM) or MβCD (10 mM) in deionized H_2O as described in Materials and Methods. The solution was then serially diluted, incubated at 25°C for 24 h, and then filtered with a 0.45-μm filter. Molar ratios were calculated as described in Materials and Methods. Values are means ± SD (n = 3). Error bars are within the markers if not apparent.

day period when the complex was stored in both plastic and glass containers at room temperature. There was 100% recovery of [^{14}C]2OHpβCD throughout the 6-day incubation in both the glass and plastic test tubes. However, there was a significant and continued loss of [^{3}H]cholesterol, resulting in a 50% decrease in solubilized cholesterol after 6 days of storage in plastic containers. No loss of cholesterol was observed in solutions maintained in glass containers. We obtained the same results with MβCD:[^{3}H]cholesterol preparations.

Effect of cyclodextrin:cholesterol complexes on cell cholesterol content

Table 1 demonstrates the ability of various concentrations of 2OHpβCD and MβCD with different CD:cholesterol molar ratios to enrich Fu5AH rat hepatoma cells with cholesterol. As can be seen, each of the CD:cholesterol solutions studied was able to effectively increase the cholesterol content of Fu5AH rat hepatoma cells after 6 h at 37°C, as compared to the control cells. Although the concentrations of 2OHpβCD and MβCD in the preparations differed, the concentrations of cholesterol complexed with the cyclodextrins were similar.

In order to examine the differences in loading capabilities between modified β-cyclodextrins, 2OHpβCD:cholesterol complexes (25 mM, 40:1) and MβCD:cholesterol complexes (5 mM, 8:1) were incubated with Fu5AH and CHO cells (**Fig. 2**). Over the 7-h incubation, there was a difference in the loading capabilities between the modified β-cyclodextrins. Although the 2OHpβCD:cholesterol complexes increased cell cholesterol content, the MβCD:cholesterol complex delivered a greater amount of cholesterol in both cell types. In Fu5AH and CHO cells exposed to MβCD:cholesterol complexes, a 2.5- to 2.75-fold enrichment of cholesterol, respectively, was seen. We observed no significant cell toxicity as measured by the release of cellular adenine (9) after an 8-h incubation as compared to control cells when Fu5AH rat hepatoma cells were enriched with cholesterol using the methods described in this study (% adenine release, control = 11.0 ± 1.0, treated = 12.7 ± 0.6, ns).

Figure 3 demonstrates that cyclodextrin-delivered cholesterol becomes metabolically available to the cells, as measured by the esterification of labeled cholesterol by ACAT. As can be seen in Fig. 3, both Fu5AH and CHO cells become enriched with labeled cholesterol over time. The free cholesterol pool remains constant as excess cholesterol is stored as esterified cholesterol. No esterification is seen in either cell type when the cells are incubated with cyclodextrin:cholesterol complexes and Pfizer ACAT inhibitor, CP-113, 818 concomitantly (data not shown), indicating that cyclodextrin-delivered cholesterol is esterified by ACAT.

In parallel experiments we determined what proportion of the cyclodextrin, if any, remained associated with the cell after washing. Fu5AH and CHO cells were incubated with [^{14}C]2OHpβCD:[^{3}H]cholesterol complexes (25 mM, 40:1) (sp act = 5.8×10^3 cpm/μg and 1.4×10^4 cpm/μg, respectively). After washing the monolayer 3× with PBS, we established that 0.4% ± 0.06 of the [^{14}C]2OHpβCD was associated with the Fu5AH cells and 0.2% ± 0.04 with CHO cells. Therefore, about 5% of the labeled cholesterol delivered to the cell monolayer during an 8-h incubation may still be associated with the cyclodextrin.

Figure 4 illustrates that variations in the level of cholesterol in either of the CD:cholesterol solutions, as described in Materials and Methods, led to modification of the cell cholesterol content, with values ranging from

TABLE 1. Ability of 2OHpβCD:cholesterol complexes and MβCD:cholesterol complexes to enrich Fu5AH rat hepatoma cells with cholesterol

	Medium		Cellular		
CD	CD:Cholesterol	Cholesterol Concentration	Cholesterol Mass	[^{3}H]Cholesterol Incorporation	% Cholesterol Esterified[a]
mM	*mol:mol*	*μg/ml*	*μg cholesterol/mg cell protein*		
Control			29.3 ± 1.4		
2OHpβCD					
10	70:1	55	60.2 ± 3.6	38.2 ± 2.9	28 ± 1.0
25	40:1	242	61.4 ± 2.6	48.1 ± 3.0	35 ± 1.5
MβCD					
1.5	20:1	29	51.4 ± 0.1	33.1 ± 7.2	35 ± 1.2
2.5	10:1	97	79.4 ± 8.5	52.6 ± 2.7	41 ± 0.1
5.0	8:1	242	79.6 ± 8.4	80.0 ± 6.9	43 ± 2.4

Fu5AH rat hepatoma cells were incubated with either 2OHpβCD:cholesterol solution of MβCD:cholesterol solution at the indicated concentrations and CD:cholesterol molar ratios. The complexes were prepared in EMEM (pH 7.2) and incubated on a monolayer of Fu5AH hepatoma cells as described in Materials and Methods for 6 h at 37°C. Total cholesterol mass was determined by gas–liquid chromatography. [^{3}H]cholesterol incorporation was determined from the cellular extracts and converted to mass based on the specific activity of the CD:[^{3}H]cholesterol solutions at the time the complexes were added to the cells. The percent of incorporated [^{3}H]cholesterol esterified was determined through thin-layer chromatography. Control indicates the cholesterol content of cells that were incubated for 6 h in EMEM alone. Data are mean ± SD of triplicate wells.

[a] % [^{3}H]esterified cholesterol/[^{3}H]total cholesterol.

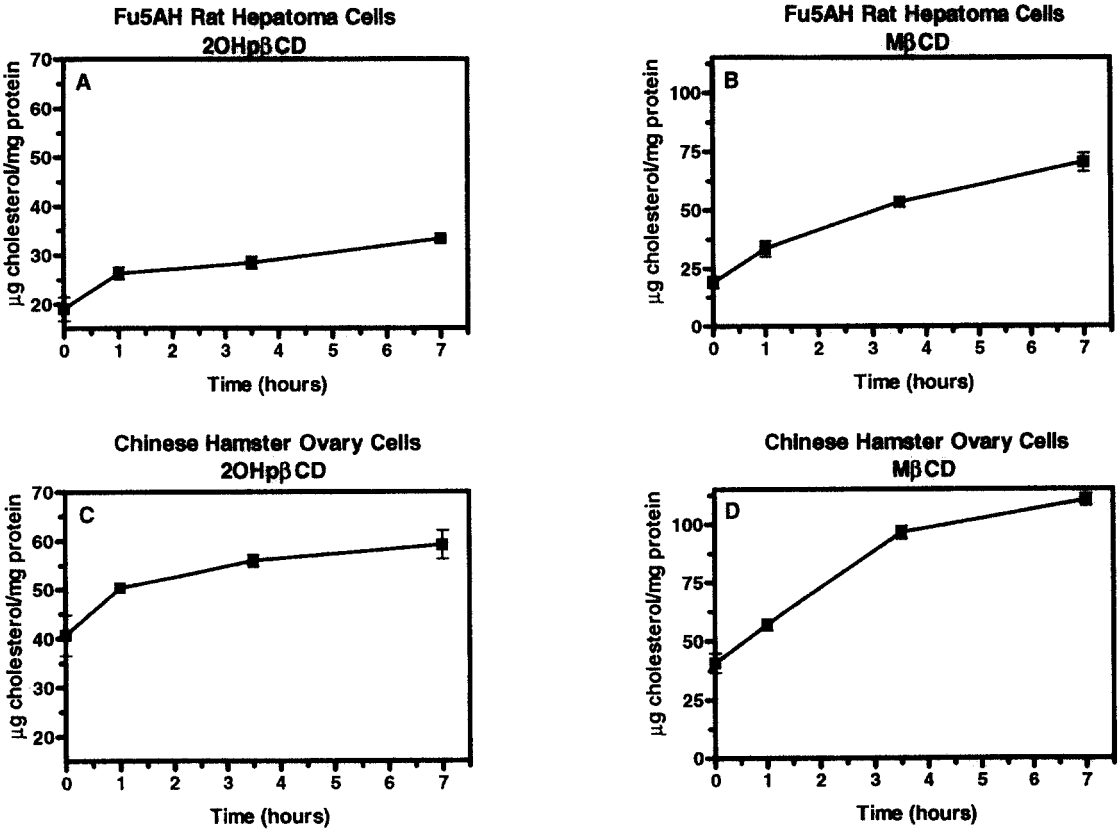

Fig. 2. Cholesterol enrichment with 2OHpβCD:cholesterol complexes (25 mM, 40:1) or MβCD:cholesterol complexes (5 mM, 8:1) in Fu5AH and CHO cells. Fu5AH rat hepatoma cells (panels A, B) and CHO cells (panels C, D) were incubated with either 25 mM 2OHpβCD:[^{3}H]cholesterol (40:1 mol:mol) or 5 mM MβCD:cholesterol (8:1 mol:mol) in EMEM (pH 7.2) for a total of 7 h at 37°C. At each timepoint, the cellular monolayer was extracted with isopropanol as described in Materials and Methods. Total cholesterol mass was determined by gas–liquid chromatography and expressed as μg cholesterol/mg cell protein. Values are means ± SD (n = 3). Error bars are within the markers if not apparent. Note the difference in the y-axis for cells treated with MβCD and cells treated with 2OHpβCD.

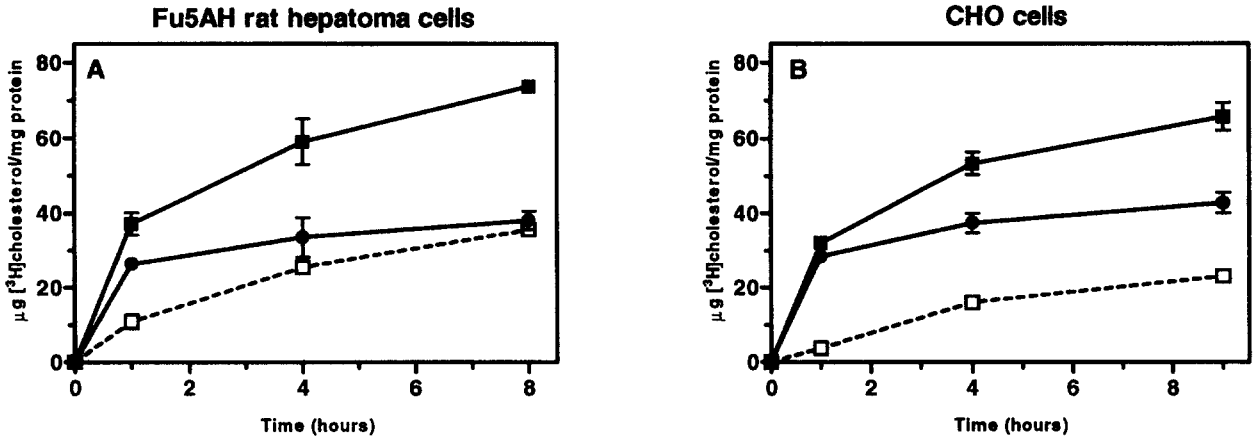

Fig. 3. [³H]cholesterol incorporation and esterification in Fu5AH and CHO cells exposed to MβCD:[³H]cholesterol complexes (5 mM, 8:1). Fu5AH rat hepatoma cells (panel A) and CHO cells (panel B) were incubated with MβCD:[³H]cholesterol complexes (5 mM, 8:1 mol:mol) in EMEM (pH 7.2) for a total of 8 h or 9 h, respectively, at 37°C. At each timepoint, the cellular monolayer was extracted with isopropanol as described in Materials and Methods. The quantitation of the incorporation of total (■) [³H]cholesterol mass from the cyclodextrin:cholesterol complexes was calculated based on the specific activity of the cholesterol in the complex. The distribution of [³H]cholesterol between pools of free (●) and esterified (□) cholesterol was determined by the recovery of radiolabeled cholesterol obtained through thin-layer chromatography. The data are expressed as µg [³H]cholesterol/mg cell protein. Values are means ± SD (n = 3). Error bars are within the markers if not apparent.

net cholesterol enrichment to net depletion. For the purpose of this experiment, 2.5 mM MβCD:cholesterol 10:1 (mol:mol) was used because its cholesterol concentration was more similar to the 25 mM 2OHpβCD:cholesterol (40:1 mol:mol) than the 5 mM MβCD:cholesterol (8:1 mol:mol) solution. As can be seen, both CD:cholesterol preparations exhibited cell cholesterol loading when the CD:cholesterol solutions were saturated with cholesterol. As the CD:cholesterol ratio increased, net efflux of cholesterol occurred, with the greatest amount of efflux occurring with cyclodextrins that contained no cholesterol (0% saturation). Cholesterol exchange (no net change in cellular cholesterol content) occurred at 50–65% saturation with the 2OHpβCD:cholesterol solutions, whereas cholesterol exchange was achieved at 75% saturation with the 2.5 mM MβCD:cholesterol solution.

DISCUSSION

Our studies indicate that, although both cyclodextrins were effective, MβCD is preferable for cellular cholesterol studies because: *1*) it is less susceptible to cholesterol precipitation upon dilution, *2*) it appears to be more effective in loading cholesterol into cells over a short period of time (8–24 h) and stimulating esterification, and *3*) it is effective for enriching and depleting cells with cholesterol (10) at much lower concentrations than 2OHpβCD. It should be noted that, although MβCD:cholesterol complexes have been shown to effectively and rapidly enrich cells with cholesterol, prolonged exposure of the cells to these complexes may lead to cell toxicity. The rapid accumulation of large amounts of free cholesterol over prolonged periods of time (>24 h) or when ACAT is blocked may circumvent the protective effect of ACAT and cause free cholesterol-associated cell toxicity (23). **Table 2** summarizes the cholesterol enrichment and esterification potential of MβCD:cholesterol complexes in a variety of cell types.

A number of procedures for introducing radiolabeled cholesterol into cells and/or enriching the cells with cholesterol mass have been described in the literature. Perhaps the most physiological approach is to supplement the medium with LDL or modified forms of LDL. If radiolabeling of the cells is desired, isotopic amounts of labeled cholesterol can be introduced into the lipoprotein. The cholesterol enrichment is most effective in macrophages and other cells expressing scavenger receptors, as the down-regulation of LDL receptors limits the extent of cholesterol enrichment otherwise. This labeling/enrichment technique usually requires many hours to days, and the exogenous cholesterol is introduced into the cells by both exchange (28, 29) and internalization (30) mechanisms, resulting in the distribution of the cholesterol among all intracellular pools. A second technique that has been used introduces cholesterol into extracellular medium containing either serum or albumin, using a solvent injection technique. However, there is evidence that, even with rela-

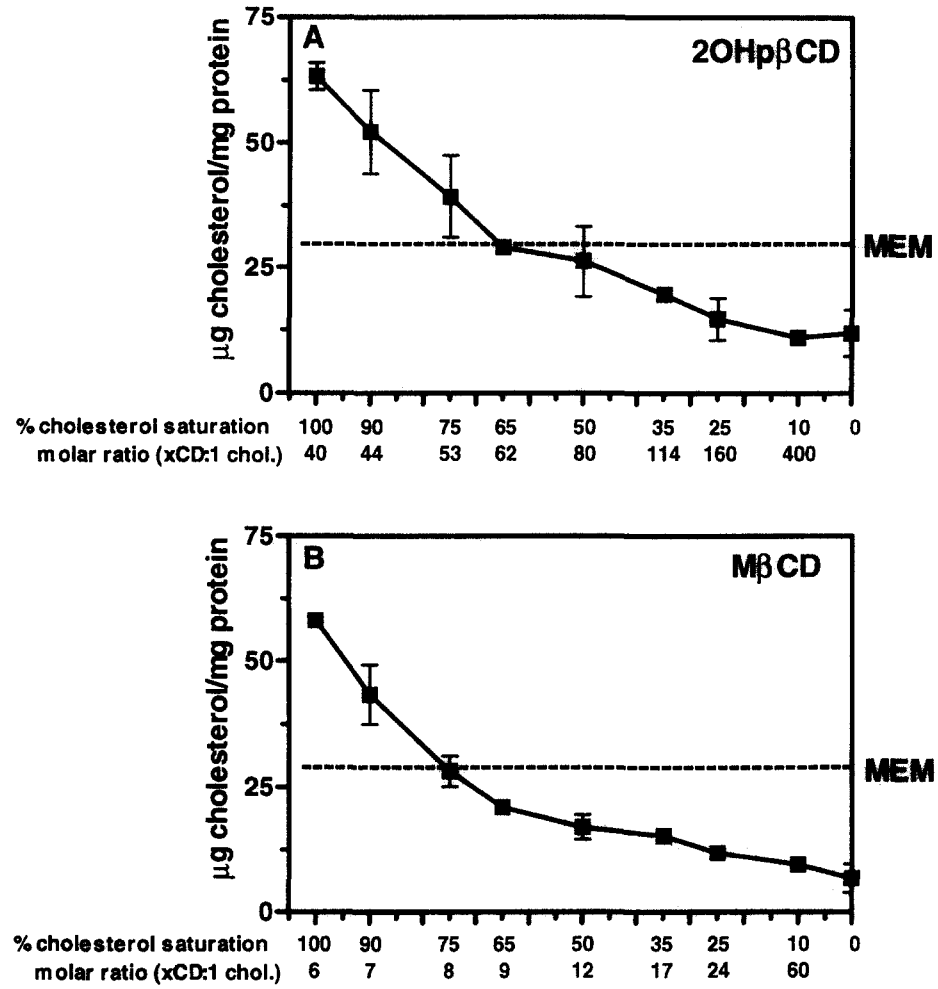

Fig. 4. Manipulation of cholesterol content using β-cyclodextrins with varying molar ratios. Fu5AH rat hepatoma cells were incubated with either 25 mM 2OHpβCD:cholesterol solution (panel A) or 2.5 mM MβCD: cholesterol solution (panel B) in EMEM (pH 7.2) with varying amounts of cholesterol for 6 h at 37°C. Total (■) cholesterol mass was determined by gas–liquid chromatography. MEM represents the total cholesterol mass (μg/mg protein) in Fu5AH hepatoma cells when incubated with EMEM for 6 h at 37°C. Data are mean ± SD of triplicate wells. Error bars are within the markers if not apparent.

tively low amounts of cholesterol, some of the added cholesterol is present in the form of microcrystals (31–33). Thus, the enrichment and/or radiolabeling that is achieved with this approach may sometimes produce an artifact with microcrystalline cholesterol attached to the cell surface.

The cyclodextrin:cholesterol system is able to deliver molecular cholesterol much more rapidly and, presumably, to a single intracellular location (the plasma membrane). Preliminary experiments using small-angle X-ray diffraction have indicated that cells incubated with cyclodextrin:cholesterol complexes do not exhibit microcrystalline cholesterol associated with the plasma membrane after washing of the monolayer. In addition, the X-ray diffraction pattern of these enriched cells demonstrates changes in membrane structure consistent with delivery of cholesterol directly to the plasma membrane (R.P. Mason and M.P. Haynes, unpublished observation). A third approach for both labeling and enriching cells in culture with cholesterol is through the use of cholesterol-enriched cholesterol/phospholipid dispersions (34). These dispersions have a cholesterol/phospholipid molar ratio greater than 1, and when incubated with cells together with serum or lipoproteins result in a considerable enrichment in cell cholesterol mass (35). The primary mechanism by which the cholesterol-rich dispersions load cells differs, depending on cell type. It involves both the exchange of molecular cholesterol from the cholesterol/phospholipid complex (36) and the internalization of cholesterol-enriched lipoproteins by receptor-mediated mechanisms (22). This cholesterol loading technique requires long incubation times (hours to days), and cannot be used to label/enrich a specific subcellular com-

TABLE 2. Ability of MβCD:cholesterol complexes to enrich cells with cholesterol

Cell Type	Cholesterol Mass[a]	% Cholesterol Esterified[b]
CHO K1		
t 0	40.67 ± 4.15	18
7 h	110.34 ± 2.62	41
Fu5AH		
t 0	19.04 ± 2.42	18
7 h	70.28 ± 3.97	52
MPM		
t 0	31.54 ± 6.92	3
8 h	61.71 ± 4.54	38
GM3468A		
t 0	39.77 ± 5.45	0
8 h	124.04 ± 24.78	16

Cells were incubated with MβCD:cholesterol complexes (5 mM, 8:1) prepared in EMEM (pH 7.2) for 7 h or 8 h at 37°C. Total cholesterol mass was determined by gas–liquid chromatography. Esterified cholesterol mass was determined as the difference between total and free cholesterol mass. Data are mean ±SD of triplicate wells.

[a] μg cholesterol/mg protein.

[b] % μg esterified cholesterol/μg total cholesterol.

partment. The availability of cyclodextrins now provides an additional approach for cell cholesterol labeling and modification of cell cholesterol content that has a number of advantages over the procedures discussed above. The preparation of the cyclodextrin:cholesterol complexes is easy and fast, and the method can also be used for the introduction of sterols other than cholesterol into the cells. Loading is extremely rapid, occurring in minutes to hours, and the extent of cholesterol enrichment is equal to, or greater than, that achieved with other techniques. In addition, although not specifically demonstrated in our present studies, the cyclodextrins probably deliver the cholesterol directly into the plasma membrane (10). Whether the cholesterol is introduced selectively into specific plasma membrane lipid domains (10, 37, 38) remains to be established. However, the newly incorporated sterol is effectively metabolized as illustrated by its rapid esterification by ACAT. Finally, as illustrated in the present study (Fig. 4), cyclodextrins provide a tool for precisely modifying cell cholesterol levels to achieve either depletion or enrichment relative to the levels occurring in cells incubated in EMEM.

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