Efflux of Newly Synthesized Cholesterol and Biosynthetic Sterol Intermediates from Cells

DEPENDENCE ON ACCEPTOR TYPE AND ON ENRICHMENT OF CELLS WITH CHOLESTEROL*

(Received for publication, August 8, 1995)

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Previous studies suggest that during sterol synthesis in cells, cholesterol and precursor sterols are transported to the plasma membrane and that this transport is stimulated by the binding of high density lipoprotein (HDL) to its putative cell surface receptor, leading to enhanced sterol efflux. Little is known about the identities of synthesized sterols subject to efflux or whether efflux of cholesterol and precursor sterols are stimulated equally by HDL. To address these issues, cells were incubated with 

\[ ^{1}H \]mevalonate and sterol acceptors, and then the labeled sterols in cells and efflux media were analyzed by high pressure liquid chromatography methods that resolved cholesterol and precursor sterols. In non-hepatic cells (Chinese hamster ovary (CHO), fibroblasts, and smooth muscle), cholesterol and multiple precursor sterols accumulated. In CHO cells, the major products were cholesterol and desmosterol, which together constituted 50% of labeled nonsaponifiable lipids. When media contained human HDL₃ (1 mg of protein/ml), the molar efflux of synthesized desmosterol was four times that of cholesterol, and the 8-h efflux of these sterols, each normalized to its own production, averaged 48 and 16%, respectively. When media contained egg phosphatidylcholine vesicles (1 mg/ml), the efflux of these sterols averaged 18 and 2.4% respectively. Thus, with both acceptors, desmosterol was the major synthesized sterol released from cells, and its efflux was substantially greater than that of synthesized cholesterol. High relative efflux of desmosterol (or a desmosterol-like sterol) occurred in all cell types and in both cholesterol-enriched and unenriched cells. These results demonstrated qualitatively similar efflux of synthesized sterols in the presence of HDL₃ and phospholipid vesicles, arguing against an absolute requirement for acceptors that interact with the HDL receptor. To probe for possible quantitative differences in the capabilities of these two acceptors, the ratios of (efflux to HDL₃)/(efflux to phosphatidylcholine vesicles) were calculated for synthesized cholesterol and desmo- sterol, plasma membrane cholesterol, and lysosomal cholesterol. In comparison to plasma membrane cholesterol, there was little or no HDL selectivity for lysosomal cholesterol or synthesized desmosterol, whereas there was a 2-3-fold selectivity for synthesized cholesterol, suggesting that the ability of HDL to enhance the efflux of synthesized sterols is a modest quantitative effect and confined to cholesterol.

In mammalian cells, the efflux of newly synthesized sterols involves translocation from the intracellular site of synthesis (the endoplasmic reticulum) to the plasma membrane, followed by sterol desorption from the plasma membrane to extracellular carriers, such as high density lipoprotein (HDL)¹ (reviewed in Johnson et al., 1991). In cells that contain normal basal levels of cholesterol, the translocation step within cells is mediated by vesicular carriers, but bypasses the Golgi apparatus and thus appears to be independent of the protein secretory pathway (Lange and Steck, 1985; Urbani and Simoni, 1990). Extensive work by Lange and colleagues (Lange and Muraski, 1987; Echevarria et al., 1990) has shown that this transport process results in the delivery of both cholesterol and biosynthetic sterol intermediates, such as lanosterol and zymosterol, to the cell surface. In work by Oram and colleagues (Mendez et al., 1991), the efflux of newly synthesized sterol has been used as a marker for the removal of internal pools of cholesterol that may contribute to cholesterol and cholesteryl ester deposition in atherosclerotic foam cells. These experiments suggest that when cellular cholesterol is elevated above normal levels, the delivery of newly synthesized sterol to the plasma membrane (and consequently the efflux of this sterol) becomes a regulated process that is stimulated when media contain acceptors that bind to the putative HDL receptor. The most important ligand for this receptor is apolipoprotein AI (apoAI), the major protein of HDL. The intracellular signaling mechanism is thought to involve diacylglycerol and protein kinase C (Mendez et al., 1991). Cyclic AMP also stimulates sterol translocation in cells, but this appears to be unrelated to HDL binding (Hokland et al., 1993). Recently, it was reported that in cholesterol-enriched cells, the delivery of synthesized sterols to the plasma membrane is sensitive to brefeldin A and proton ionophores, suggesting participation of the Golgi apparatus in HDL-regulated sterol translocation (Mendez, 1995).

The diversity of biosynthetic sterols that find their way to the plasma membrane has led to concern about the identities of these sterols and whether they are released from cells with equal efficiency. The data addressing these issues are limited

¹ The abbreviations used are: HDL, high density lipoprotein; apo-HDL, depleted lipoproteins of human HDL; LDL, low density lipoprotein; HPLC, high pressure liquid chromatography; TLC, thin layer chromatography; GC, gas chromatography; MS, mass spectrometry; PC, phosphatidylcholine; SUV, small unilamellar vesicle; BSA, bovine serum albumin; DLP, degradized calf serum protein; NSL, nonsaponifiable lipids; CHO, Chinese hamster ovary.

* This work was supported by National Institutes of Health Program Project Grant HL22633. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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and somewhat contradictory. In mouse peritoneal macrophages, analysis by reverse-phase thin layer chromatography indicated that the major sterol produced from \([{}^{1}H]\)mevalonolactone was desmosterol and that this sterol and cholesterol were released to HDL with similar efficiency (Aviram et al., 1989). In the recent studies of Hokland et al. (1993), an analysis of biosynthetic products in fibroblasts by reverse-phase high pressure liquid chromatography suggested a different pattern, in that the major product in cells was cholesterol, whereas the major products released to HDL were polar sterols with retention times somewhat different from that of desmosterol. In this case, there were significant differences in the efficiency of release of different sterols. In other work with fibroblasts, Echevarria et al. (1990) reported that zymosterol was a major product of sterol synthesis and that its efflux from glutaeraldehyde-fixed cells to diluted blood plasma was about two times more efficient than that of newly synthesized cholesterol.

The objective of the present studies was to provide a more complete quantitative understanding of the efflux of newly synthesized cholesterol in comparison to the major sterol intermediates that had been reported to accumulate in cells during typical labeling procedures. The critical technical aspect of the experiments was to analyze samples using a chromatographic method capable of separating cholesterol and the major intermediates of sterol synthesis. To satisfy this need, all samples were analyzed by a reverse-phase high pressure liquid chromatography (HPLC), which was connected in series with a flow-through liquid scintillation counter to provide a sensitive, high resolution profile of the distribution of radiolabeled sterols in each experimental sample. The results confirm that in a variety of non-hepatic cells (CHO, fibroblasts, and smooth muscle cells) several sterol intermediates more polar than cholesterol accumulate and are available for efflux. A product comigrating with desmosterol (and conclusively identified as this sterol in CHO cells) was the major biosynthetic sterol released from non-hepatic cells. Additional experiments addressed the dependence of cholesterol and desmosterol efflux on the type of acceptor provided in the medium and on whether cells are pre-enriched with exogenous cholesterol.

**Experimental Procedures**

**Materials—**Tissue culture supplies, solvents, and most other reagents were from sources noted previously (Johson et al., 1990, 1991). Delipidized bovine serum protein (DLP) was prepared from bovine calf serum (Rothblat et al., 1976; Capriotti and Laposta, 1987). DL-[\(^{2-}\)H]Mevalonic acid lactone (1 C/mm) was from Amersham Corp. Compound 58035 was a gift from Dr. J. John Heider (Sandoz). Triparanol was a gift from Dr. Ekkehard Bohme (Marion Merrell Dow Inc.). Mevinolin compound 58035 was a gift from Dr. John Heider (Sandoz). Triparanol was a gift from Dr. Peter Gillies (DuPont Merck Pharmaceuticals). [\(^{3}H\)]Acetic acid (sodium salt, 3.6 C/mm) was from DuPont NEN. For the addition of radiolabeled precursor to incubation medium, an appropriate aliquot of the precursor stock solution in organic solvent (as supplied by the manufacturer) was taken to dryness under nitrogen and then redissolved in aqueous medium. [\(^{3}H\)]Mevalonate and [\(^{3}H\)]acetate typically were used at concentrations of approximately 20 and 50 \(\mu\)Ci/ml, respectively. Media were not supplemented with unlabeled "carrier" of either precursor. Low density and high density lipoproteins (LDL and HDL) were prepared from fresh human plasma by ultracentrifugation (Hatch and Lees, 1968). Egg PC small unilamellar vesicles (PC-SUV) and reconstituted PC-apoHDL discs (PC/protein ratio = 2.1, w/w) were prepared by sonication (Barenholz et al., 1977; Lund-Katz and Phillips, 1984). Acceptor preparations were dialyzed against the appropriate tissue culture medium and filter sterilized (0.45-\(\mu\)m pore size) before use. CHO-K1 and HepG2 cells were from seed cultures obtained from the American Type Culture Collection (Rockville, MD). CHO-met18b2 cells were from a seed culture provided by Dr. Jerry Faust (Tufts University, Boston, MA). This CHO variant expresses a plasma membrane transporter for mevalonate and as a result is unusually efficient at incorporating exogenous mevalonate into cholesterol. In all cells, cholesterol was added at a concentration of 25 mg/ml for 1 h with 0.2% BSA to allow desorption of any loosely adherent lipids, and then incubated with labeled [\(^{3}H\)]acetate with or without sterol acceptor. To enrich cells with free (unesterified) cholesterol, the incubation with DLP was followed by a 1-day incubation in medium containing 50 \(\mu\)g/ml of LDL, 100 \(\mu\)g/ml of HDL and 25 \(\mu\)g/ml of Egg PC liposomes (Arbogast et al., 1976). 1% fetal bovine serum, 0.2% bovine serum albumin (BSA), and 1 \(\mu\)g/ml Sandoz compound 58035. All subsequent media contained compound 58035 to prevent sterol esterification. After the 1-day enrichment period, cells were reseeded, incubated 1 h with 0.2% BSA to allow desorption of any loosely adherent lipids, and then incubated with labeled [\(^{3}H\)]acetate with or without sterol acceptor. Control (unenriched) cells were treated identically except that during the enrichment period the medium did not contain LDL and the liposomes were cholesterol-free.

For HPLC, the column dimensions were 4.6 \(\times\) 250 mm (diameter \(\times\) length), the packing was 5-\(\mu\)m C-18 Spherisorb (Isco), the solvent was acetonitrile-isopropyl alcohol (3:1, v/v) flowing at 1 ml/min, and the column was prepared in 100 \(\mu\)l. The effluent was directed to a UV absorbance detector and a liquid scintillation counter. The cell volume for liquid scintillation counting was 0.5 ml and the data-sampling interval was 6 s. Software provided by Radiomatic was used for peak integration and the preparation of side-by-side plots of UV absorbance and \(^{3}H\) radioactivity. There was a 1-min delay between the UV and \(^{3}H\) signals, which was compensated for by the software. The retention times reflect the times of appearance of peaks at the UV cell. The retention times of cholesterol were 16-19 min, varying somewhat from day to day. The retention times of commercial standards relative to cholesterol were as follows: 25-hydroxycholesterol, 0.35; 4-cholesten-3-one, 0.69; desmosterol, 0.72; 7-dehydrocholesterol, 0.80; squalene, 0.84; lanosterol (two peaks), 0.82 and 1.14. The presence of two components was observed in several commercial lanosterol preparations. This may be due to the presence of both authentic lanosterol (relative retention time 0.82) and dihydrolanosterol (1.14). The reproducibility of relative retention times was \(\pm\)0.01.

Thin layer chromatography (TLC) of NSL was performed with glass-backed Silica Gel G plates using a development solvent of hexane-ethyl acetate (70:30, v/v). Mass amounts of lipids were visualized by staining with iodine vapor. Distributions of radiolabel were imaged using a Radiomatic TLC-600 gas-ionization thin layer plate scanner. R\(_f\) values of standards can be calculated from data in Fig. 1.

Digitonin precipitation was performed as described by Sperry and
Webb (1950) using 1 mg of unlabeled cholesterol as a "carrier" and 2.5 ml of 0.5% (w/v) digitonin to effect precipitation of sterols. After rinsing, the precipitated sterols were dissociated from the digitonide complex by treatment with hot dimethyl sulfoxide and extracted into petroleum ether (Issidorides et al., 1962) for analysis by HPLC.

Combined gas chromatography (GC)/electron-impact mass spectrometry (MS) was performed without derivatization of sterols using a Hewlett-Packard model 59970 GC-MS, as described previously (Fischer et al., 1989). The sterols were partitioned using a DB17 capillary column (J & W Scientific, Folsom, CA) with a column oven temperature program (235–265 °C, 5 °C/min) to improve resolution. For each sterol, the mass spectrum is that of the major peak eluting from the GC column.

In-vial liquid scintillation counting and assays for protein, cholesterol, and phospholipid were as described previously (Johnson et al., 1990, 1991).

In a given experiment, all incubations were performed in at least triplicate. Values are the means of these replicate determinations. Uncertainties are ± 1 S.D. Statistical significance was assessed by Student's t test, with p < 0.05 as the criterion of significance.

Fig. 1. HPLC analysis of NSL and cholesterol TLC fraction from [3H]acetate-labeled CHO-K1 cells. CHO-K1 cells were incubated 24 h in medium containing BSA (0.2%), compound 58035 (1 µg/ml), and [3H]acetate (200 mCi/ml), and then NSL were prepared from the cells. A portion of the NSL along with unlabeled standards was analyzed by thin layer chromatography (stationary phase: Silica Gel G; mobile phase: hexane-ethyl acetate, 70/30, v/v). Panel A shows the migration of the standards (as determined by staining with iodine vapor) and the distribution of 3H radioactivity (as determined by scanning with a gas-ionization counter). Abbreviations: orig., TLC origin; 25HC, 25-hydroxysteroid; chol., cholesterol; lanost., lanosterol; squa., squa-lene; front, TLC solvent front. The major peak of radioactivity from the TLC plate (co-migrating with cholesterol standard) was extracted with chloroform-methanol (1:1, v/v). Panels B and C show the HPLC analysis of the total NSL and the cholesterol TLC fraction, respectively.
RESULTS

To establish methods for the analysis of newly synthesized sterols, NSL were prepared from CHO cells that had been incubated with \(^{3}H\)acetate for 24 h, and then TLC and reverse-phase HPLC were compared as methods of detecting and quantifying \(^{3}H\)-labeled cholesterol and other products in the NSL. By TLC, most of the nonsaponifiable \(^{3}H\) co-migrated with cholesterol (Fig. 1A). However, by HPLC the labeled NSL were seen to consist of about 20% cholesterol plus three other major products that were somewhat more polar than cholesterol (Fig. 1B). When the “cholesterol” TLC fraction was isolated and analyzed by HPLC, a profile very similar to that of the total NSL was obtained (Fig. 1C). Thus, the “cholesterol” TLC fraction was a mixture of products, and most of the radioactivity in this TLC fraction was not associated with cholesterol when analyzed by HPLC. These results are consistent with previous work by Echevarria et al. (1990), Burki et al. (1987), and Hokland et al. (1993), who have also reported that the incubation of mammalian cells with nonsterol precursors of cholesterol leads to the accumulation of a diversity of products, some of which are not resolved from cholesterol using typical TLC methods. Thus, in studies of this type it appears to be essential that a method with high resolving ability, such as reverse-phase HPLC, be used to obtain an accurate assessment of the synthesis and efflux of individual sterols. The use of TLC alone could yield misleading results. The data reported in this paper are based entirely on the reverse-phase HPLC analysis of total NSL, as illustrated in Fig. 1B.

To determine whether the four major products resolved by HPLC were sterols, labeled NSL were subjected to digitonin precipitation, and then the precipitable sterols were recovered from the digitonide complex and compared to total NSL by HPLC. The results indicated quantitative precipitation of cholesterol and product 2, but only partial precipitation of products 1 and 3. Thus, product 2 was identified conclusively as a 3β-hydroxysterol (Haslam and Klyne, 1953). Product peaks 1 and 3 may be poorly precipitable sterols or may be mixtures of precipitable and nonprecipitable products.

To examine both the synthesis and efflux of newly synthesized sterols, CHO cells were incubated simultaneously with labeled precursor (\(^{3}H\)acetate or \(^{3}H\)mevalonate) and an extracellular sterol acceptor, and then NSL from cells and media were analyzed by HPLC. Over several experiments, qualitatively similar data were obtained using either acetate or mevalonate as the precursor. HPLC profiles from an experiment using \(^{3}H\)mevalonate are shown in Fig. 2. In this experiment, the medium also contained either BSA alone or BSA plus HDL \(_3\) (at a concentration of 1 mg of protein/ml). Under both conditions (Fig. 2, A and B, respectively), the cells accumulated radiolabel in cholesterol and in the three polar products noted in Fig. 1 (peaks labeled 1–3). When the incubation medium contained HDL \(_3\), there was significant efflux of all four products (Fig. 2C). Interestingly, it did not appear that the products were released in proportion to their accumulation in cells. Product 2 in particular was clearly subject to disproportionately high efflux.

Because of its interesting efflux behavior and the strong evidence that it was a sterol, additional attention was given to the identification of product 2. Its retention time on HPLC (0.72–0.73 relative to cholesterol) was consistent with it being

![Fig. 2. HPLC analysis of biosynthetic sterols in CHO cells and efflux medium.](http://www.jbc.org/)

The efflux of \(^{3}H\)NSL to medium containing just BSA was only 0.75%, making HPLC analysis of this medium impractical. Panel A, NSL from cells incubated with BSA in the absence of HDL. Panel B, NSL from cells incubated with BSA and HDL \(_3\). Panel C, NSL from medium containing BSA and HDL \(_3\).
The difference in A-dienesterol (e.g. plus HDL3, and egg phosphatidylcholine vesicles) were examined in CHO cells under three acceptor conditions (BSA alone, BSA plus HDL3. The results demonstrated steady production and total NSL, cholesterol, and desmosterol in CHO cells incubated with HDL3. The results showed that the medium sample was deemed to have too little radioactivity to justify HPLC analysis of even a pooled sample. Each incubation was performed in triplicate. However, at early time points, it sometimes was necessary to pool triplicate samples of medium NSL in order to have enough radioactivity for a single useful HPLC profile. For this reason, data are plotted without error bars. When three independent determinations of a value were obtained, S.D. values typically were 5-15%. Data points missing at the 0.5-h time point indicate that the medium sample was deemed to have too little radioactivity to justify HPLC analysis of even a pooled sample. Each panel shows the recovery of the indicated component in cells (circles), in medium (triangles), and in cells + medium (squares).

To determine whether the results on synthesis and efflux of sterols obtained with CHO cells were representative of other cells, an experiment comparing several different cell types (CHO, human fibroblasts, rabbit smooth muscle cells, human HepG2 hepatoma cells) was performed. When the different cell types were incubated for 8 h with \(^{3}H\)acetate and no sterol acceptor in the medium (Fig. 4, left panels), similar complex sterol profiles were obtained in the four non-hepatic cell types (CHO-met18b2, CHO-K1, GM3468A fibroblasts, and rabbit smooth muscle cells), consisting mostly of cholesterol and two or three other major products with retention times less than that of cholesterol. In all cases, sterol 2 (co-migrating with desmosterol) was prominent. In contrast, HepG2 cells accumu-

### Table I: Mass Spectral Analysis of Sterol 2 from CHO Cells

| M/z Fragment | Percentage Abundance Relative to Base Peak |
|--------------|------------------------------------------|
| Sterol 2     | Desmosterol                               | Zymosterol |
| 384          | 23                                        | 20         | 9          |
| 369          | 31                                        | 29         | 25         |
| 351          | 15                                        | 18         | ND         |
| 300          | 28                                        | 26         | 15         |
| 299          | 17                                        | 18         | ND         |
| 271          | 100                                       | 84         | 100        |
| 253          | 16                                        | 20         | ND         |
| 69           | 87                                        | 96         | 53         |

* The 351 m/z fragment results from the loss of H\(_2\)O and the C19 methyl group from the sterol's A-ring.
* ND, not detected.
* Allylic fragmentation between carbons 22 and 23 occurs with \(\Delta^\text{24} \)
  unsaturated side chain sterols, along with methyl group loss. This fragmentation correspondingly proceeds with a proton abstraction from the D-ring, which is much less evident for the \(\Delta^\text{24} \)
  zymosterol.
* Fission of a \(\Delta^\text{24} \)
  Unsaturated side chain between carbons 17 and 20 in a concurrent abstraction of two protons from the D-ring. In addition to this side chain loss, the 253 m/z ion is also characterized by the loss of H\(_2\)O.

Fig. 3 illustrates the time courses of synthesis and efflux of total NSL, cholesterol, and desmosterol in CHO cells incubated with HDL\(_3\). The results demonstrated steady production and efflux of NSL and both sterols over 24 h. The disproportionately high release of desmosterol in comparison to cholesterol was apparent at all time points.

Tables II and III provide a compilation of data from several experiments in which the synthesis and efflux of sterols in CHO cells under various three acceptor conditions (BSA alone, BSA plus HDL3, and egg phosphatidylcholine vesicles) were examined after an 8-h incubation period. The data in Table II demonstrate that under all three conditions, cholesterol and desmosterol together accounted for approximately half of newly synthesized NSL in cells, and that the two sterols were present in roughly equal amounts, although in this regard there was considerable variability from experiment to experiment. The data in Table II also show that cholesterol and desmosterol were available for efflux to both HDL\(_3\) and PC vesicles and that in both cases the contribution of desmosterol to the newly synthesized sterol efflux was three to four times the contribution of cholesterol (i.e. cholesterol was only about 10% of the biosynthetic NSL in the efflux medium, whereas desmosterol was 30-42% of the biosynthetic NSL in the medium). In Table III, the efflux data are re-expressed in fractional units (i.e. efflux of a given component normalized to its accumulation in cells + medium during the 8-h incubations). These data show that with HDL\(_3\), the efflux of desmosterol was about three times more efficient than the efflux of cholesterol. The difference in efflux efficiencies appeared to be even greater with PC vesicles (6-9-fold).

![Fig. 3. Time courses of efflux of newly synthesized NSL and sterols from CHO cells to HDL\(_3\). Conditions were as described in Fig. 1, except that the incubation time varied from 0.5 to 24 h. Sterol identifications were based on HPLC retention times relative to cholesterol. Each incubation was performed in triplicate. However, at early time points, it sometimes was necessary to pool triplicate samples of medium NSL in order to have enough radioactivity for a single useful HPLC profile. For this reason, data are plotted without error bars. When three independent determinations of a value were obtained, S.D. values typically were 5-15%. Data points missing at the 0.5-h time point indicate that the medium sample was deemed to have too little radioactivity to justify HPLC analysis of even a pooled sample. Each panel shows the recovery of the indicated component in cells (circles), in medium (triangles), and in cells + medium (squares).](http://www.jbc.org/)

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To determine whether HDL₃ was particularly efficient in comparison to PC-SUV at promoting efflux from specific cellular compartments, fibroblast and CHO cell data on sterol efflux from different cellular pools were compiled and compared. Data were available for synthesized sterols from the present work and for the plasma membrane and lysosomal cholesterol pools from previous studies performed under similar conditions (see Table V references). For each sterol pool, the data were expressed as the ratio of efflux to HDL₃/efflux to PC-SUV, using acceptor concentrations of 1 mg of protein/ml and 1 mg/ml, respectively (mol/mol efflux ratios, Table V). Then, to probe for HDL-specific influences on the delivery of internal sterol to the plasma membrane, the ratio for plasma membrane cholesterol was set equal to 1, and the other ratios were re-expressed accordingly (normalized efflux ratios, Table V). These calculations show that in both CHO cells and fibroblasts, the efflux ratio for lysosomal cholesterol was identical to that for plasma membrane cholesterol. In CHO cells, the ratios for synthesized desmosterol and cholesterol were greater than plasma membrane cholesterol by factors of 1.2 and 2.9, respectively. In GM3468A fibroblasts, the corresponding values were...
1.3 and 2.2, respectively. These results indicate that synthesized sterol 2 (desmosterol) was released from cells with a low acceptor specificity, similar to that governing the efflux of lysosomal cholesterol (Johnson et al., 1990, 1991). In contrast, the efflux of newly synthesized cholesterol, although much slower than that of sterol 2 under all conditions tested (Tables II-IV), was enhanced by HDL₃, suggesting that the delivery of synthesized cholesterol to the plasma membrane may be more efficient in the presence of HDL₃. Using data from Table IV, it can also be calculated that the enrichment of cells with choles-
Effects of cellular cholesterol enrichment on efflux of newly synthesized sterols from CHO-K1 cells and human GM3468A fibroblasts

All incubation media were supplemented with 1 μg/ml of Sandoz compound 58035 to prevent cellular cholesterol esterification (Ross et al., 1984). Cells were enriched with cholesterol by incubation for 1 day in medium containing fetal bovine serum (1%), BSA (0.2%), human LDL (50 μg protein/ml), and cholesterol-rich liposomes (cholesterol/phosphatidylcholine = 2.2 mol/mol, 92 μg of phosphatidylcholine/ml) (Johnson et al., 1988). Control cells were treated identically, except the medium did not contain LDL and liposomes did not contain cholesterol. Following these treatments, cells were rinsed and incubated 1 h at 37 °C in medium containing 0.2% BSA (to allow desorption of any unincorporated lipids), and then incubated in medium containing [3H]acetate (200 μCi/ml) and either PC-SUV (1 mg/ml) or HDL3 (1 mg of protein/ml) and BSA (0.2%). Synthesis and efflux of sterols were quantified as in previous experiments. Control and cholesterol-enriched CHO cells contained 19 ± 2 and 38 ± 6 μg of free (unesterified) cholesterol (FC)/mg of protein, respectively. The corresponding values in fibroblasts were 20 ± 4 and 88 ± 18 μg of FC/mg of protein, respectively. Control CHO cells in this experiment were identical to those of experiment 1 in Tables II and III. Efflux is expressed in units of fraction released/24 h. Because of inefficient incorporation of [3H]acetate into NSL of cholesterol-enriched cells, it was not possible to obtain reliable estimates of efflux in these cells at earlier time points. Values in parentheses indicate efflux in comparison to the corresponding values for control cells.

| Cell type | Treatment | Acceptor | Total NSL Cholesterol Sterol 2 |
|-----------|-----------|----------|-------------------------------|
| CHO-K1    | Control   | HDL3     | 0.43 ± 0.05                   |
| CHO-K1    | FC-rich   | HDL3     | 0.33 ± 0.01                   |
| CHO-K1    | Control   | PC-SUV   | 0.20 ± 0.02                   |
| CHO-K1    | FC-rich   | PC-SUV   | 0.14 ± 0.01                   |
| GM346BA   | Control   | HDL3     | 0.61 ± 0.02                   |
| GM346BA   | FC-rich   | HDL3     | 0.43 ± 0.04                   |
| GM346BA   | Control   | PC-SUV   | 0.35 ± 0.01                   |
| GM346BA   | FC-rich   | PC-SUV   | 0.14 ± 0.01                   |

HDL enhancement of efflux of newly synthesized cholesterol in comparison to other sterol pools

Data on efflux of synthesized sterols from the current studies and data on efflux of plasma-membrane and LDL-derived lysosomal cholesterol from previous studies were expressed as the ratio (efflux to HDL3)/(efflux to PC-SUV), when [HDL3] = 1 mg of protein/ml, and [egg PC-SUV] = 1 mg/ml. Each of these molar efflux ratios then was normalized to the molar ratio obtained for plasma membrane cholesterol in order to assess any enhancement of efflux of internal sterol by HDL.

| Cell type | Sterol pool | (Efflux to HDL3)/(Efflux to SUV) | Refs. |
|-----------|-------------|---------------------------------|-------|
| CHO-K1    | Synthesized cholesterol | 7.9 | 2.9 | Table IVb |
| CHO-K1    | Synthesized desmosterol | 3.3 | 1.2 | Table IVb |
| CHO-K1    | Plasma membrane cholesterol | 2.7 | 1.0 | Table IVb |
| CHO-K1    | Lysosomal cholesterol | 2.7 | 1.0 | Table IVb |
| GM346BA   | Synthesized cholesterol | 3.3 | 2.2 | Table IVb |
| GM346BA   | Synthesized sterol 2 | 2.0 | 1.3 | Table IVb |
| GM346BA   | Plasma membrane cholesterol | 1.5 | 1.0 | Johnson and Reinhart (1994) |
| GM346BA   | Lysosomal cholesterol | 1.5 | 1.0 | Johnson and Reinhart (1994) |

* The mol/mol efflux ratio divided by the mol/mol efflux ratio for plasma membrane cholesterol.
* Control cells (unenriched) were prepared as described in Table IV, except the incubation time was 8 h. As shown in Fig. 4, initial rates of synthesized sterol release were largely sustained for at least 8 h.
* Unpublished data on efflux of plasma membrane and LDL-derived lysosomal cholesterol from W. J. J ohnson. Methods were as described in Johnson et al. (1990). The incubation time was 4 h.
* The ratios were calculated from data on efflux of plasma membrane and LDL-derived lysosomal cholesterol in Table I of Johnson and Reinhart (1994). The incubation time was 4 h.

The enhanced efflux of synthesized cholesterol in the presence of HDL3 did not require prior elevation of cellular cholesterol content.

To provide an additional test of the selective enhancement of cholesterol efflux by HDL, we compared the abilities of PC-SUV and a reconstituted PC-apoHDL discoidal complex to deplete cholesterol and desmosterol mass from CHO-K1 cells. In this case, the cells were grown in lipid-free medium (Fig. 5) so that they contained only synthesized sterols. In addition, to stabilize the levels of cholesterol and desmosterol in the system during efflux, the cells were treated with a combination of compound 58035, mevinolin, and triparanol (inhibitors of acyl-CoA:cholesterol acyltransferase, hydroxymethylglutaryl-CoA reductase, and sterol 24-reductase, respectively). The mass data indicated that both PC-SUV and PC-apoHDL were able to cause net depletion of cellular desmosterol, with the apoHDL acceptor being two to three times more efficient than SUV (Fig. 5B). For cellular cholesterol (Fig. 5A), the fold difference in acceptor efficiency was much greater, with PC-apoHDL removing 60% of cholesterol after 24 h and SUV removing 10% or less (a change that was not statistically significant but does not contradict the more precise radio-tracer data in Tables III and IV). Thus, the mass data were consistent with the efflux patterns described in Table V and provide independent confirmation of the selective enhancement of endogenous cholesterol release by HDL.

**DISCUSSION**

The results of these studies suggest that sterol synthesis in non-hepatic cells is accompanied by the accumulation of both cholesterol and several polar sterol intermediates. As implied by efflux to extracellular acceptors, the intermediates do not remain confined to the endoplasmic reticulum or other internal...
Efflux of Newly Synthesized Sterols

Fig. 5. Depletion of CHO cell cholesterol and desmosterol mass by PC-SUV and PC-apoHDL acceptors. CHO-K1 cells were grown to approximately 50% confluence in 35-mm tissue culture wells with medium containing DLP (5 mg/ml) and Sandoz compound 58035 (1 μg/ml), and then incubated 2 days with the same medium also supplemented with triparanol (1 μM), an inhibitor of sterol 24-reductase, to induce accumulation of easily measurable amounts of desmosterol. At this time, the cells were confluent and were incubated overnight with 1.5 ml/well of medium containing DLP, compound 58035, triparanol, and mevinolin (1 μg/ml), to stabilize the levels of desmosterol and cholesterol in the cells. After this overnight incubation, depletion of cellular sterol was examined under three efflux conditions: 1) BSA (0.2%), 2) BSA + PC-apoHDL (500 μg of PC/ml), and 3) PC-SUV (1 mg/ml). Exposure to the three drugs was continued under all three efflux conditions. Cells were analyzed for content of cholesterol and desmosterol by gas-liquid chromatography after 0, 8, and 24 h of efflux. Results are plotted as sterol content/mg cell protein versus incubation time under the different conditions.

organelles, but rather are delivered along with cholesterol to the outer leaflet of the plasma membrane. During efflux, cholesterol and the intermediates are not released from cells with equal efficiency. In particular, a sterol with the chromatographic properties of desmosterol (and conclusively identified as this sterol in CHO cells) is released from cells several times more efficiently than is newly synthesized desmosterol. As a result of the substantial production of this sterol and its tendency to undergo rapid efflux, the major synthesized sterol released from non-hepatic cells appears to be desmosterol (or a desmosterol-like sterol) rather than cholesterol. This finding holds for both cholesterol-depleted cells (in which cholesterol synthetic activity is high) and for cholesterol-enriched cells (in which synthetic activity is low), and for both lipoprotein (HDL₃) and non-lipoprotein (PC-SUV) acceptors. However, the results indicate a significant degree of enhancement of the efflux of newly synthesized cholesterol in response to incubation with HDL. This enhancement is not seen for newly synthesized desmosterol or for the lysosomal pool of cholesterol. The enhancement suggests the possibility of regulation of the efflux of newly synthesized cholesterol by HDL and other biological acceptors that are thought to participate in reverse cholesterol transport.

Implications Regarding Sterol Transport in Cells and Regulation of Sterol Efflux—Lange and colleagues (Lange and Muraski, 1987; Echevarria et al., 1990; Lange et al., 1991) have investigated the transport and distribution of newly synthesized sterols in fibroblasts using a variety of methods, including subcellular fractionation and the availability of sterols to exogenous cholesterol oxidase. The results of their work indicate substantial delivery of multiple sterol intermediates to the plasma membrane. This conclusion is supported by the present results demonstrating substantial efflux of sterol intermediates to extracellular acceptors, a process that requires the delivery of sterols from the endoplasmic reticulum to the plasma membrane. Echevarria et al. (1990) reported that a major sterol intermediate in human fibroblasts was zymosterol and that the efflux of this sterol from glutaraldehyde-fixed cells to diluted blood plasma was twice as efficient as the efflux of newly synthesized cholesterol. On reverse-phase HPLC, zymosterol migrates much like desmosterol (Hansbury and Scallen, 1980). Thus, the desmosterol-like sterol detected in fibroblasts in the present studies may have been zymosterol.

Previous studies exploring the acceptor-specificity of biosynthetic sterol efflux suggested that in cholesterol-enriched cells the efflux was stimulated by the presence of acceptors that bind to the putative HDL receptor (Oram et al., 1983; Aviram et al., 1989). Thus, HDL₃ was reported to promote efflux of newly synthesized sterols, whereas acceptors such as PC vesicles and nitroxylylated HDL, which cannot bind to the receptor, were much less effective (Sliote et al., 1987). Limited data have been provided on the composition of sterols released from cells. Tabacik et al. (1991) used thin layer chromatography methods to characterize the synthesis and efflux of biosynthetic sterols in smooth muscle cell cultures and reported the preferential release of sterol precursors in comparison to cholesterol during the incubation of cells in diluted calf serum. The described precursors appeared to consist predominantly of late intermediates (i.e. C-27 dienes), but precise identification of the sterols was not performed. Aviram et al. (1989) reported that in human monocyte-derived macrophages, most biosynthetic sterol that accumulated and was released from cells co-migrated with desmosterol on reverse-phase thin layer chromatography. The efflux of this sterol to HDL₃ was reported to be similar to that of biosynthetic cholesterol. Hokland et al. (1993) reported that in human fibroblasts the biosynthetic sterols consisted of cholesterol and multiple unidentified components with HPLC retention times somewhat different from that of desmosterol. The fractional efflux of the intermediates to HDL₃ was greater than that of cholesterol, although the efflux of both cholesterol and the other sterols was stimulated by cAMP. These previous results indicate that polar intermediates are the predominant biosynthetic sterols released from cells and suggest that the efflux behavior of these sterols resembles that of biosynthetic cholesterol. Conclusive identification of the intermediates has not been provided. Nor has the efflux of each sterol been quantified separately.

The present results add to previous findings by clearly identifying desmosterol as the main biosynthetic sterol released from CHO cells and by indicating that either desmosterol or a structurally similar sterol is the main biosynthetic sterol released from other extrahepatic cells, including fibroblasts. In contrast to the conclusions of previous studies, we found that the sterols synthesized in cholesterol-enriched cells are available for efflux to an acceptor (PC-SUV) that does not bind to the HDL receptor (Table IV). Thus, the efflux of synthesized sterols does not require acceptors that bind to the putative HDL re-
The methodology of reverse-phase HPLC in combination with continuous liquid-scintillation monitoring of the HPLC effluent provided a detailed quantitative profile of newly synthesized sterols undergoing efflux. The ability to quantify individual sterols using this methodology provided evidence that HDL selectively enhances the efflux of synthesized cholesterol (Table V). In contrast to the results of Aviram et al. (1989), this enhancement appears to be confined to synthesized cholesterol and is not observed for synthesized desmosterol or for LDL-derived lysosomal cholesterol (Table V). The enhancement may be due to the interaction of HDL with the putative HDL receptor, although its occurrence in both cholesterol-enriched and unenriched cells argues against this explanation. Additional experiments will be needed to determine the basis for this enhancement. These experiments could involve direct tests of the diacylglycerol and cAMP signaling mechanisms, which have been reported to stimulate sterol translocation to the plasma membrane.

The greater efflux of desmosterol in comparison to cholesterol may have been due to greater delivery of desmosterol to the plasma membrane, greater desorption of desmosterol from the plasma membrane to extracellular acceptor particles, or a combination of such differences. Analysis of the sterol profile in isolated plasma membranes and studies on sterol efflux from isolated plasma membranes will be used to resolve this question. Relevant to this issue are studies of Clejan and Bittman (1984) reporting that the efflux of desmosterol from the surface membrane of Mycoplasma gallisepticum to egg phosphatidylcholine vesicles is approximately 30% slower than the efflux of cholesterol. If this finding also applies to the plasma membrane of mammalian cells, it would suggest that the greater fractional efflux of desmosterol relative to cholesterol may be due to more rapid delivery of desmosterol to the plasma membrane. Consistent with this possibility, Lange et al. (1991) reported that the transport of newly synthesized zymosterol to the plasma membrane in fibroblasts was two times faster than that of newly synthesized cholesterol.

Related Considerations—A potential non-biological explanation for the accumulation of both cholesterol and several polar products during sterol synthesis is that cholesterol was the only major product attributable to metabolic activity and that the polar compounds were the result of uncontrolled oxidation of samples during the analytical workup. This explanation is considered very unlikely in view of the results obtained with HepG2 cells (Fig. 4), in which cholesterol was the main biosynthetic sterol product, even though the analytical workup in this case was different from that used with other cell types in the same experiment.

The results of these experiments indicated substantial efflux of several nonsaponifiable lipid products other than cholesterol (e.g. Fig. 2C, peaks 1–3). Major attention was focused on desmosterol (peak 2) in this article because of the major contribution of this sterol to the efflux of the newly synthesized sterols and because we were able to provide a conclusive identification of this sterol in CHO cells. It will be of interest in future studies to establish the identities of the other intermediates that accumulate during sterol synthesis and to further explore their availability for efflux.

The presence of low concentrations of sterol intermediates in blood plasma is well documented in the work of Miettinen and colleagues (Bjorkhem et al., 1987; Vanhanen et al., 1993). Previously, the presence of these intermediates in blood has been attributed largely to leakage from the liver (Bjorkhem et al., 1987). However, in the present studies non-hepatic cells appeared to be much better sources of sterol intermediates than either HepG2 human hepatoma cells (Fig. 4) or FuSAH rat hepatoma cells (data not shown). In these liver-derived cell lines, cholesterol was the major sterol synthesized and released to extracellular acceptors. These findings may indicate that non-hepatic tissues are major sources of the sterol intermediates that are found in blood plasma. This possibility is consistent with the conclusion reached by Dietschy et al. (1993) that non-hepatic tissues contribute substantially to sterol synthesis in mammals, based on the results of in vivo metabolic studies. Another interesting contrast derived from the present results is the large efflux of sterol intermediates from non-hepatic cells in comparison to the very low concentrations of intermediates relative to cholesterol in blood plasma (Bjorkhem et al., 1987). This disparity may imply unusually efficient mechanisms for the clearance of sterol intermediates from plasma. There appears to be very little direct information relating to this question or to the subsequent metabolism of these sterols after uptake into cells. These topics deserve further investigation.

Acknowledgments—We are grateful to Christine E. Ackerman and Aimee E. Christian for excellent technical assistance. We also thank Dr. John Helder (Sandoz Corporation) for supplying compound 58–035, Dr. E. B. Bohme (Marion Merrell Dow Research Institute), Dr. K. Torstila, I., and Miettinen, T. A. (1993) J. Lipid Res. 34, 1535–1544.
