Rat Adrenal Uptake and Metabolism of High Density Lipoprotein Cholesteryl Ester*

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Metabolism of high density lipoprotein (HDL) cholesteryl ester (CE) by cultured rat adrenal cells was studied. Addition of [3H]CE-HDL to cells pretreated with adrenocorticotropic hormone in lipoprotein poor media resulted in a time- and concentration-dependent accumulation of [3H]cholesteryl ester and production of [3H]cholesterol and [3H]eorticosterone. HDL-CE metabolism could be described as the sum of a high affinity ([HDL-cholesterol], max = 16 µg/ml) and low affinity ([HDL-cholesterol], max > 70 µg/ml) process. [3H]Cholesterol was found both intracellularly and in the media. Accumulation of [3H]cholesteryl ester could not be attributed to uptake and re-esterification of unesterified cholesterol since addition of Sandoz 58-035, an inhibitor of acyl coenzyme A:cholesterol acyltransferase, did not prevent ester accumulation. Moreover, addition of chloroquine did not inhibit cholesteryl ester hydrolysis indicating that hydrolysis was not lysisosomally mediated.

Aminoglutethimide prevented conversion of [3H]CE-HDL to steroid hormones but did not inhibit [3H]cholesteryl ester uptake. Cellular accumulation of [3H]cholesteryl ester exceeded accumulation of 125I-apo-proteins 5-fold at 1 h and 35-fold at 24 h indicating selective uptake of cholesteryl ester moieties. We conclude that rat adrenal cells possess a mechanism for selective uptake of HDL cholesteryl esters which provides substrate for steroidogenesis. These results constitute the first direct demonstration that cholesteryl esters in HDL can be used as steroidogenic substrate by the rat adrenal cortex.

Cholesterol is an obligate intermediate in the synthesis of steroid hormones. Uptake of extracellular lipoprotein cholesterol is an important source of substrate in most steroidogenic tissues (1). It is now apparent that at least three distinct mechanisms exist for cellular uptake of lipoprotein cholesterol. These include receptor-mediated endocytosis (2), aqueous diffusion (3, 4), and a poorly understood nonendocytotic process for selective uptake of cholesteryl esters (5-8). Specific receptors which recognize apoB (9), apoE (10), or modified apoB (11, 12) mediate endocytosis of intact lipoprotein particles. Lipoproteins lacking these apoprotein determinants do not deliver cholesteryl esters by receptor-mediated endocytosis.

Net cellular uptake of unesterified cholesterol can occur by aqueous diffusion or by a process, perhaps involving direct lipoprotein cell contact but no more energetically favorable than aqueous diffusion (3, 13). Because of the low aqueous solubility of cholesteryl esters, cellular uptake of cholesteryl esters does not occur by aqueous diffusion (3).

A third process for net cholesterol movement is exemplified by rat adrenal uptake of HDL cholesterol (5-8, 14). In the rat adrenal cortex HDL is the preferred lipoprotein source of steroidogenic substrate cholesterol (1). Uptake of HDL cholesteryl esters cannot be attributed to receptor-mediated endocytosis or to aqueous diffusion (7, 14). Lipoprotein particles lacking apoproteins recognized by endocytosed receptors, such as human HDL, are nonetheless able to enhance rat adrenal steroid production by delivery of substrate cholesterol. Recent studies from our laboratory suggest that unesterified cholesterol accounts for only a portion of the steroidogenic substrate cholesterol taken up by this process, since esterification of HDL cholesterol only partially diminishes HDL-dependent steroid hormone synthesis (14). In addition, reports by Pittman, Glass and their colleagues (6, 7) as well as Leitersdorf et al. (8) using HDL labeled with cholesteryl ethers, a non-degradable marker for cholesteryl esters, indicate that adrenal, hepatic, and other tissues of both rodents and humans can selectively accumulate cholesteryl esters considerably in excess of HDL apoprotein. Nestler et al. (15) using HDL doubly labeled in the cholesterol and cholesteryl ester moieties have shown that rat ovarian granulosa cells utilize cholesteryl esters for synthesis of progesterone. Verschoor-Klootwyk et al. (16) have also presented evidence for cholesteryl ester uptake from HDL although their studies did not preclude uptake mediated by apoE.

The inverse relationship between HDL cholesterol (17, 18) or its major apoprotein A-I (19) and risk of coronary heart disease has stimulated study of cellular HDL metabolism. Rodent steroidogenic tissues provide a novel and useful system for the study of cellular HDL metabolism. In these tissues, HDL metabolism is hormonally regulated and produces a physiologic response, increased steroidogenesis (5, 20). Current evidence suggests that one way in which HDL may protect against coronary heart disease is through "reverse cholesterol transport," the removal of cholesterol from peripheral tissues and subsequent delivery to the liver for excretion (21, 22). A thorough understanding of HDL metabolism at the cellular level is essential to complete understanding of the anti-atherogenic actions of HDL. Much has been learned about HDL metabolism by steroidogenic and other tissues through the use of nonhydrolyzable cholesteryl ethers (6-8).

Since cholesteryl ethers cannot be hydrolyzed, they do not provide a suitable probe for following further the metabolism

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The abbreviations used are: HDL, high density lipoprotein; ACTH, adrenocorticotropic; AG, aminoglutethimide; 1.125B, serum of density greater than 1.125 g/ml; CE, cholesteryl ester; LDL, low density lipoprotein; TLC, thin layer chromatography; LPP, lipoprotein poor media.

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of accumulated cholesteryl esters. Moreover, because the
others cannot be metabolized they may have unappreciated
pharmacologic effects intracellularly, although they are me-
tabolic turnover intravenously (6,8). We have undertaken
the current studies to directly examine the metabolism of
HDL cholesteryl esters in rat adrenocortical cells. While
previous studies have clearly documented that adrenal tissue
selectively accumulate cholesteryl esters and esters (6–
8), only indirect evidence suggests that cholesteryl esters in
HDL may be utilized by the adrenal for steroid substitution.
Since no definitive proof has yet been presented, the first
objective of our studies was to determine if cholesteryl esters in
HDL could be utilized for corticosterone production. As a
basis for determining the molecular mechanisms responsible
for cholesteryl ester uptake, we have further characterized the
uptake process. Finally, since only unesterified cholesteryl
can undergo side chain cleavage, the committed step in ste-
roidogenesis, we have examined cell-mediated hydrolysis of
HDL cholesteryl esters.

**EXPERIMENTAL PROCEDURES**

**Lipoprotein Preparation, Labeling, and Characterization.** To ex-
amine the metabolism of HDL cholesteryl esters independently of the
apoB receptor pathway, we required HDL which was (a) free of
apoE; (b) contained radioabeled unesterified cholesterol, and (c) contained
no radiolabeled unesterified cholesterol, and (d) retained native ste-
roidogenic activity. Human HDL was chosen since it is essentially
free of apoE. In previous studies employing purified HDL (14) not
subjected to heparin-Sepharose chromatography, apoE was undetect-
able by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and
present in less than 0.3% of total protein by radioimmunoassay.
Moreover, our previous studies have shown that human HDL does
not enhance rat adrenal steroidogenesis through the low density
lipoprotein pathway (5,14,24). In the current studies we have, in
some cases, employed human HDL further freed of apoE by passage
over a heparin-Sepharose affinity column (25).

Human serum HDL was labeled with [3H]cholesteryl-oleate by a
modification of the method of Thomas and Rudel (26). To enhance
the efficacy of labeling and prevent modification of HDL, we have
employed serum of d > 1.125 g/ml (hereafter termed 1.125B) rather
than whole serum. Plasma was collected in EDTA (1 mg/ml) from
normal human volunteers and centrifuged at d = 1.125 g/ml for 18 h,
at 8°C using a Beckman Ti-60 fixed angle rotor. The upper half of
the screw top tube was aspirated and discarded. The lower half,
(1.125B), was dialyzed against at least four changes of 50 volumes of
0.1 M NaCl, 10 mM EDTA (0.2 g/ml) for 6 h. Previous studies have shown that incubating 1.125B for 18 h at 37°C causes loss of
steroidogenic activity as a result of cholesterol esterification.
To ensure retention of native HDL properties, we therefore inactivated lecithin cholesterol acyltransferase (27) by incubating 1.125B under
nitrogen at 36°C for 30 min prior to labeling or further purification.

Subsequently, inactivated 1.125B was labeled by incubation under
nitrogen at 37°C for 18–24 h in screw top glass tubes which had [3H]
cholesteryl-oleate dried onto 100–200 mg of Celite 545 (Supelco Inc.,
Belleville, PA). Incubations were performed with 1.125B rather
than purified HDL in order to provide a source of cholesteryl ester
exchange protein. Usually 5–7 ml of 1.125B was incubated with 100–
200 mCi of [3H]cholesterol-oleate which had been purified by TLC.
At the end of the labeling incubation, celite was removed by centrif-
ugation and millipore (0.2 micron) filtration. In preliminary experi-
ments, the degree of labeling observed with purified HDL was less
than 10% of that seen with 1.125B. Typically, 40 to 60% of the label
was incorporated and specific activities ranged from 12500 to 75000
dpm/µg cholesteryl. At the completion of the labeling incubation greater than 99% of the incorporated label co-migrated with
cholesteryl oleate on TLC.

To determine if labeling altered the lipoprotein, fluorometric steroid
production by ACTH/LPP-pretreated rat adrenal cells incubated 24 h
with [3H]cholesterol oleate labeled or unlabeled 1.125B, added at
equal total cholesterol concentration, was compared. In six compari-
sons of five different preparations, the mean steroid production for
labeled materials was not different from zero (r = 0.9, p = 0.837) indicating that the labeling procedure has preserved metabolic HDL
activity. All labeled HDLs and 1.125B preparations were tested and used only if normal steroidogenic activity was retained. Our previous
studies (14) have shown that in the presence of LPP, fetal calf serum, 1.125B and purified HDLs support steroidogenesis equally well. We have used
1.125B and 1.125B labeled by sequential ultracentrifugation as previously described (14) and freed of apoE by column chromatography on heparin-Sepharose
(25). Lipoprotein purity was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (28). No apoE was detected either
before or after column purification. Purified HDLs and human HDL
were iodinated using a modification of the iodine monochloride pro-
cedure described by Bilheimer et al. (29) as previously described (30).
Lipoprotein poor human or fetal calf serum were prepared by cen-
trifugation at d = 1.215 g/ml for 48 h, as previously described (14).
Unlabeled human HDL (1.125 < d < 1.215 g/ml) and low density
lipoprotein (1.019 < d < 1.055 g/ml) were purified by sequential
density ultracentrifugation as previously described (14).

**Cell Preparation and Culture.** Suspensions of adrenocortical cells
were prepared from glands excised from 21–42-day-old female Sprague-Dawley rats by collagenase-DNase digestion using a modific-
ation of the method of Kowal and Fiedler (31). Cells were originally described (14). Cells were initially plated on multilwell dishes (Falcon
3847) in F-12/Dulbecco’s modifed Eagle’s medium (1:1) supple-
mented with 10% fetal calf serum (HyClone) plus penicillin (100
units/ml) and streptomycin (100 µg/ml) plus 3% bovine brain growth supplement (standard media). The medium was replaced after 24 h, as described by McCaillier and Hornsby (32). The bovine brain growth supplement was prepared by the method of Gospodarowicz et al. (33) omitting the final column purification. In some cases, 2% (v/v) Ultsor-G
(IBC Biotechnics, Savage, MD) was substituted for bovine brain growth supplement. In preliminary experiments, addition of either
the bovine growth supplement or Ultsor-G had no acute effect on
steroidogenesis or metabolism of [3H]cholesterol-ester in HDL (data
not shown).

Except where noted, we have used confluent monolayer cultures which
were pretreated for 48 h with ACTH (10−9 M) in lipoprotein
poor media. This approach was chosen to enhance the utilization of
extracellular cholesterol substrate, based on our earlier studies (5).

**Assays.** Cholesterol and cholesteryl ester were measured enzymat-
ically using reagents supplied in kit form by Boehringer Mannheim
(No. 124087, West Germany). Steroid hormone production was mea-
sured fluorometrically using a modification of the method of Kowal
and Fiedler (31), as previously described (15). Corticosterone was
used as the standard, and the results are expressed in terms of corticosterone equivalents, the amount of corticosterone required
to produce a fluorescent signal equal to the unknown. Protein was
determined by the method of Lowry et al. (35) using bovine serum
albumin as a standard. Corticosterone was isolated from methylene chloride extracts of media by TLC on aluminum-backed silica gel plates (Silica Gel-60, MCB Reagents, Gibbstown, NJ) developed with toluene/acetone (80:20). Lipids and steroids, extracted from media with chlo-
roform/methanol according to Bligh and Dyer (36), were similarly
isolated by TLC on silica gel plates. The isolated products were cut
from the sheets and added directly to Safety-Solve (Research Prod-
ucts International, Mount Prospect, IL) scintillation mixture prior to
counting using a Rackbeta liquid scintillation counter (LKB Instru-
ments, Gaithersburg, MD). Cell cholesterol was extracted with
isopropanol as described by Johnson et al. (13). Degradation of
appoinptes in HDL was determined by measurement of total media
121I which did not precipitate when made 10% in trichloroacetic acid
for 60 min at 4°C (37). Statistical analyses were performed using
EpiStat, public domain software for IBM microcomputers.

**Materials.** Heparin-Sepharose was obtained from Pharmacia
LKB Biotechnology Inc. [3H](1,2)-cholesteryl-oleate was purchased from Du Pont-New England Nuclear. [3H]Cholesteryl-
oleate was chromatographed on TLC plates prior to use.

**RESULTS**

To determine if cholesteryl esters in HDL could serve as
steroidogenic substrate, HDLs (1.125 < d < 1.215 g/ml) labeled with
[3H]cholesteryl-oleate was incubated with primary con-

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fluently treated with ACTH in lipoprotein poor media for 48 h. The following two types of labeled HDL preparations were used: (i) partially purified HDLs consisting of plasma of d > 1.25 g/ml (3H-CE-1.125B) and (ii) fully purified HDLs prepared from 1.125B by centrifugation and freed of apoE by heparin-Sepharose chromatography (apoE-free 3H-CE-HDL). In separate experiments employing eight different cell preparations and five different labeled HDL preparations, with concentrations of HDL cholesterol ranging from 4.8 to 100 μg/ml, from 1.5 to 17.5% of the added counts were converted to steroid products (Table I). Similar results were observed with partially purified 3H-CE-HDL (3H-CE-1.125B) (Table I, experiments 1 and 2) and apoE-free-3H-CE-HDLs (Table I, experiments 3–8). Within single experiments, the percent of added counts converted to steroid products decreased as the concentration of HDL increased.

Control studies were performed to confirm that counts recovered from the media were products of cell-mediated steroidogenesis. Production of fluorogenic steroids (panel A) and radiolabeled steroid products (panel B) for a representative control experiment are shown in Fig. 1. In the absence of added lipoprotein (column 1) or in the absence of cells (column 3) no radioabeled steroid products were formed. In the absence of added lipoprotein (column 1), cells produced 1.50 ± 0.01 μg of corticosterone equivalents/well. Addition of 3H-CE-HDL (column 2) increased steroid production 3.3 times and resulted in conversion of 3.4% of the added counts to steroid products. Addition of aminoglutethimide (AG) (column 4) or cyanide/flouride (column 5) inhibited both fluorogenic and radiolabeled steroid production. Addition of a 5-fold excess of unlabeled HDL (column 6) enhanced fluorogenic steroid production greater than that produced by addition of labeled HDL alone (cf. column 2) but inhibited conversion of 3H-CE from HDL to steroid products.

Incubation of 3H-CE-HDL with cells resulted in cholesteryl ester hydrolysis. When introduced into the media, greater than 99% of the HDL radioactivity comigrated on TLC with cholesteryl esters (Fig. 2). Only background counts were recovered from TLC plates at the position of unesterified cholesterol and corticosterone. The percent of counts recovered from the media was unesterified cholesterol following incubation for 24 h in the absence of cells increased from 0.34 ± 0.03% to 1.34 ± 0.05% (p < 0.001) while the percent of counts recovered as cholesteryl ester decreased accordingly (99.5 ± 0.05% to 98.4 ± 0.07%, p < 0.001). No change in the percent of added counts recovered as steroid products occurred (0.210 ± 0.01% unincubated sample). In three similar cell-free incubations, from 0.9 to 1.3% of the added counts were recovered as unesterified cholesterol. In contrast, in the presence of cells, counts recovered as unesterified cholesterol increased to 2.89 ± 0.08% (p < 0.001 cf. unincubated sample) and counts recovered as cholesteryl ester decreased to 92.5 ± 0.53% (p < 0.001 cf. unincubated sample) of counts recovered from the media. Counts recovered as steroid product increased to 4.65 ± 0.47%, p < 0.001, cf. unincubated sample) of added counts. Thus, the percent of added 3H-CE converted to steroid products is severalfold greater than the percent of unesterified counts recovered from unincubated material or material incubated in the absence of cells.

Following incubation of 3H-CE-HDL with cells, 3H-unesterified cholesterol was found both cell associated and in the media as well. Total cell-mediated hydrolysis was considered to be the sum of media and cell unesterified cholesterol less the amount of unesterified cholesterol generated in the absence of cells. To determine if hydrolysis of HDL cholesteryl esters is lysosomally mediated, we compared the amount of 3H-CE from HDL hydrolyzed by cells incubated in the presence and absence of chloroquine. The amount of 3H-CE from HDL hydrolyzed by the cells in the presence of chloroquine was the same or even greater than in its absence (Fig. 3). Although, no inhibition of 3H-CE hydrolysis was observed, chloroquine (100 μM) did inhibit fluorogenic steroid production by 42.5%, an observation previously noted by others (38).

### Table I: Synthesis of steroid hormones from HDL cholesteryl esters

| Experiment no. | [HDL-C] μg/ml | Specific activity HDL-C dpm | Sterol dpm | % added dpm in sterol | Fluorogenic steroid due to HDL* μg/well | Estimated % product derived from HDL-CEb |
|---------------|--------------|---------------------------|------------|-----------------------|-------------------------------------|---------------------------------------|
| HDL = 1.125B  | 35           | 74,838                    | 66,860     | 6.4                   | 1.97                                | 45.3                                  |
| 2             | 71           | 74,838                    | 72,987     | 3.4                   | 2.12                                | 46.0                                  |
| HDL = apoE-free HDL no. | 25 | 31,845                    | 23,767     | 7.5                   | 1.05                                | 70.8                                  |
| 4             | 25           | 31,845                    | 12,907     | 4.1                   | 1.08                                | 37.6                                  |
| 5             | 50           | 12,524                    | 4,883      | 2.0                   | 1.08                                | 36.2                                  |
| 6             | 25           | 12,524                    | 3,196      | 2.6                   | 1.04                                | 24.5                                  |
| 7             | 4.8          | 74,688                    | 25,116     | 17.5                  | 0.42                                | 80                                    |
| 9.6           | 74,678       | 45,609                    | 17.0       | 0.65                  | 100                                 |                                       |
| 19.2          | 18,669       | 14,632                    | 10.2       | 1.20                  | 65                                  |                                       |
| 40            | 18,669       | 24,299                    | 8.1        | 1.80                  | 72                                  |                                       |
| 80            | 18,669       | 35,212                    | 5.9        | 2.60                  | 75                                  |                                       |
| 123           | 12,487       | 6,589                     | 9.7        | 0.51                  | 91                                  |                                       |
| 24            | 12,847       | 6,589                     | 5.3        | 0.70                  | 74                                  |                                       |
| 35            | 12,847       | 7,738                     | 4.3        | 0.97                  | 62                                  |                                       |
| 100           | 12,847       | 7,887                     | 1.5        | 0.99                  | 62                                  |                                       |

*a* Difference in total fluorogenic steroid production in the presence and absence of HDL.

*b* Total steroid hormone production estimated to be twice the amount determined by fluorescence (29).
and specific receptor-mediated degradation of human 125I-LDL by 81% (triplicate determination from triplicate experiments, data not shown).

To determine if cell-accumulated [3H]cholesterol esters arose from re-esterification of hydrolyzed HDL cholesteryl esters, we measured cell accumulation of [3H]-CE in the presence of Sandoz 58-035, an inhibitor of acyl-coenzyme A:cholesterol-acyltransferase (38). Preliminary studies were performed to determine the concentration of Sandoz 58-035 required to inhibit cholesteryl esterification in rat adrenal cells. When cells were incubated with [3H]cholesterol, added in ethanol, in the presence of increasing doses of Sandoz 58-035 and AG, to prevent conversion of accumulated [3H]cholesterol to steroid products, dose-dependent inhibition of cholesteryl esterification was observed (Fig. 4). Fifty % inhibition occurred at a concentration less than 0.1 μg/ml. To determine if inhibition of cholesteryl esterification was due to general-
Fig. 5. Effects of Sandoz 85-035 on HDL-dependent and independent steroidogenesis. A, primary monolayer cultures of rat adrenocortical cells which had not been pretreated were incubated for 18 h in fresh complete media (Vme = 0.5 ml) with increasing doses of Sandoz 58-035 (added in 0.005 ml of MeSO). Steroid production was measured fluorometrically. B, primary monolayer cultures of rat adrenocortical cells, pretreated for 72 h with ACTH/LPP (Vme = 0.5 ml) were subsequently incubated for 18 h in fresh LPP media supplemented with HDL (50 μg/ml total cholesterol) and increasing doses of Sandoz 58-035 (added in 0.005 ml of MeSO). Steroid production was measured fluorometrically (mean and S.D. of triplicate measures, typical of three such experiments). Cort, corticosterone equivalents.

Fig. 6. Effects of Sandoz 58-035 on cell accumulation of 3H-cholesteryl ester from HDL. Primary monolayer cultures of rat adrenocortical cells were pretreated for 48 h with ACTH/LPP and subsequently incubated for 18 h with 3H-CE-1.125B (30 μg/ml total cholesterol, specific activity = 42,400 dpm/μg of cholesterol) in LPP media with AG (800 μM) and Sandoz 58-035 (1 μg/ml). The cells were thoroughly washed and cell-associated unesterified cholesterol and cholesteryl esters extracted and separated by TLC. The mean and S.D. of triplicate determinations from duplicate experiments are shown. FC, free cholesterol; CE, cholesteryl ester.

Fig. 7. Effects of aminoglutethimide on uptake and metabolism of HDL cholesteryl esters. Primary confluent monolayer cultures of rat adrenocortical cells, pretreated for 48 h with ACTH (10−7 M) in LPP media, were incubated for 18 h with ACTH (10−7 M), 3H-CE-HDL (25 μg of total cholesterol/ml) with and without AG (0.8 mM) or unlabeled HDL (150 μg of total cholesterol/ml) as indicated. Total cell associated 3H-cholesterol (FC + CE) were extracted from the cell pellet by isopropyl alcohol. Media 3H-steroids were extracted with methylene chloride and isolated by TLC. The means ± S.D. of triplicate determinations from a single experiment representative of three such experiments are shown.

Fig. 8. Time course of cellular accumulation of HDL cholesteryl esters. Confluent monolayer cultures of rat adrenocortical cells, pretreated for 48 h with ACTH (10−7 M) in LPP media, were incubated for the indicated times with 3H-CE-HDL (25 μg of total cholesterol/ml), ACTH (10−7 M), and AG (0.8 mM). The cells were thoroughly washed and cell associated 3H-cholesterol extracted with isopropyl alcohol and counted. The mean and S.D. of triplicate determinations from a single experiment representative of three such experiments is shown.

Net cellular metabolism of [3H]cholesteryl ester from HDL is comprised of the sum of 3H-steroid production, total cellular accumulated 3H-CE and 3H-unesterified cholesterol plus media 3H-unesterified cholesterol, in excess of that found in cell-free controls. To determine if addition of AG stimulated cell uptake of HDL cholesteryl esters or simply redirected them from steroidogenesis into alternative pathways, net HDL-CE metabolism was measured in the presence and absence of AG. Addition of AG prevented conversion of 3H-CE from HDL to 3H-steroids but did not diminish total cell metabolism (Fig. 7). In the absence of AG, addition of excess unlabeled HDL with 3H-CE-HDL inhibited total cell metabolism of 3H-CE (Fig. 7).

Cellular accumulations of 3H-sterols from HDL was time (Figs. 8 and 9) and concentration (Fig. 10) dependent. When 3H-CE-1.125B was added to ACTH/LPP-pretreated cells, there was an initial rapid increase followed by a prolonged
Parental monolayer cultures of rat adrenocortical cells, pretreated for 48 h with ACTH (10^{-7} M) in LP5 media, were incubated for the indicated times with ACTH (10^{-7} M), AG (0.8 mM), and 3H-CE-1,125I-HDL. The cells were thoroughly washed and extracted with isopropyl alcohol. The cell-associated 125I remaining in the cell extract and the extracted total [3H]cholesterol were counted. The mean and S.D. of triplicate determinations from a single experiment representative of three similar experiments are shown.

**Fig. 9.** Comparison of the accumulation of [3H]cholesterol and 125I-protein from HDL by rat adrenocortical cells with time. Primary confluent monolayer cultures of rat adrenocortical cells, pretreated for 48 h with ACTH (10^{-7} M) in LP5 media, were incubated for the indicated times with ACTH (10^{-7} M), AG (0.8 mM), and 3H-CE-1,125I-HDL. The cells were thoroughly washed and extracted with isopropyl alcohol. The cell-associated 125I remaining in the cell extract and the extracted total [3H]cholesterol were counted. The mean and S.D. of triplicate determinations from a single experiment representative of three similar experiments are shown.

**Fig. 10.** Concentration-dependence of fluorescent steroid production, 3H-steroid production, and cell 3H-sterol accumulation. A. primary confluent monolayer cultures of rat adrenocortical cells, pretreated for 48 h with ACTH (10^{-7} M) in LP5 media, were incubated for 18 h with ACTH (10^{-7} M) and increasing concentrations of 3H-CE-HDL. Separate multwell plates (24 x 15 mm) were used to measure total fluorogenic steroids (O) and 3H-steroid (Δ) production in the absence of AG or 3H-sterol (●) accumulation in the presence of AG (0.8 mM). Total fluorogenic steroid production was estimated from measurements of corticosterone equivalents as described in the text. Micrograms of 3H-sterol accumulation and 3H-steroid production have been calculated from total dpm divided by the specific activity of cholesteryl ester in added HDL. Each response curve in A was individually normalized by dividing all values in each curve by the maximum value for that parameter. The maximum value for each parameter occurred at the maximum HDL concentration tested. Inset: double reciprocal plot of the normalized values for all parameters.

The effects of increasing HDL concentration on (i) fluorescent steroid production, (ii) production of 3H-labeled steroid products, and (iii) cell-associated [3H]cholesterol and cholesteryl esters was determined by incubating 3H-CE-1,125I-HDL at increasing concentrations with ACTH/LP5-pretreated cells. Cell-associated counts were determined in the presence of AG, to prevent conversion to corticosterone while AG was omitted for measurements of fluorescent and 3H-steroids. All three parameters exhibited similar dependence on HDL concentration (Fig. 10A). To more directly compare the dose response curves for the three outcome parameters, each curve was normalized by dividing all values by the maximum observed value which in each case occurred at an HDL concentration of 100 μg of cholesterol/ml. The normalized dose response curves were virtually coincident (Fig. 10B). The response curves were complex, increasing steeply at low HDL concentrations but much more gradually at higher HDL concentrations. When the combined normalized response curves were analyzed graphically, a nonlinear double reciprocal plot was observed (inset Fig. 10B). The double reciprocal plot could be resolved into two linear components. The concentrations of HDL required to half-maximally stimulate the two processes suggested by this method of analysis were 16.3 and 54.9 μg of HDL cholesterol/ml. The latter is consistent with our earlier studies (5) which suggests that half-maximal HDL concentration for enhanced fluorogenic steroid production and cell cholesteryl accumulation is at least 70 μg of HDL cholesterol/ml.

**DISCUSSION**

Previous studies of adrenal HDL metabolism, by us (5, 15, 40) and others (6–8, 41–51) demonstrate that HDL delivers sterioiogenic substrate cholesterol and replenishes cellular cholesterol stores by a non-endocytotic process. We have recently presented evidence that both unesterified cholesterol and cholesteryl esters from HDL can be utilized for sterioiogenic substrate (14). Additional support for a role of cholesteryl esters arises from the studies of Glass et al. (6, 7) and Stein et al. (7, 8) showing that the rat adrenal cortex selectively accumulates cholesteryl ester, a cell-retained nonhydrolyzable cholesteryl ester analog, from HDL. This elegant approach, most useful for quantitating tissue cholesteryl ester uptake in vivo, does not permit investigation of subsequent
in intracellular cholesteryl ester metabolism. In addition, ether analogs could interfere with or modify normal ester metabolism (52). Fortunately, in a closed *in vitro* system, trapped analogs are not essential for studying ester metabolism since both intracellular and extracellular cholesteryl ester metabolites can be quantitatively recovered. Thus, the current studies, which were undertaken to directly characterize rat adrenal metabolism of cholesteryl esters, have employed HDL labeled exclusively in the cholesteryl ester moiety.

To ensure that results we obtained employing radiolabeled cholesteryl esters accurately reported the activity of native HDL cholesteryl esters, we sought to demonstrate that the labeling procedure did not alter HDL activity and that the labeled and unlabeled cholesteryl esters behaved identically. Comparing enhancement of ACTH-stimulated steroid production by labeled and unlabeled HDL provides an unique opportunity to evaluate the effects of labeling on HDL function since HDL-enhanced steroidogenesis is one of only a few established physiologic functions of HDL. Two observations indicate that the labeling procedure did not alter HDL structure. First, labeled and unlabeled HDL, when added to cultured cells at equivalent cholesterol concentrations, produce identical increases in ACTH-stimulated steroidogenesis. Secondly, the dose response curves for enhanced fluorogenic steroid production and production of $^3$H-steroids exhibited an identical dependence on HDL concentration. The latter observation further indicates that incorporated [$^3$H]cholesteryl esters behave the same as endogenous cholesteryl esters. Since we wished to examine apoE and apoB,E receptor-independent cholesteryl ester uptake we chose to use human HDL, which is free of apoE, apoE, and other apoproteins recognized by endocytosed receptors. To ensure that HDL-associated apoE was not responsible for cholesteryl ester uptake we often used HDL preparations freed of apoE by heparin-Sepharose chromatography. We could detect no significant differences in the metabolism of $^3$H-CE-1.125B, $^3$H-CE-HDL, or $^3$H-CE-apoE-free HDL. The labeling procedure and lipoprotein preparations used in this study thus provided a suitable and convenient means of obtaining labeled HDL which (i) retains normal function, (ii) is labeled solely in the cholesteryl ester moiety, and (iii) are not endocytosed.

Since the current studies were designed to observe the contribution of apoB,E receptor-mediated endocytosis to total HDL-CE metabolism we elected to use human HDL since rat HDL cannot be entirely freed of apoE. Dyer and Curtiss (53) have recently reported that addition of human rat apoE-rich HDL to cultured rat ovarian cells selectively stimulates progesterone production while apoE-free HDL stimulated both progesterone and androgen production. These investigators have proposed that apoE inhibits luteinizing hormone stimulated increases in 17-hydroxylase, 17–20 lyase enzyme activity. In earlier studies (5), we could detect no differences in total fluorogenic steroid production by cells incubated with human or rat HDL added at equal cholesterol concentrations nor could we detect, by TLC, differences in the types of steroids produced in the presence and absence of human HDL. The possibility remains that rat HDL which contains apoE or human HDL containing apoE may alter the types of adrenal steroids produced when compared with apoE-free HDL. Studies are currently underway to answer this intriguing question.

The studies reported here directly demonstrate that cholesteryl esters from HDL can be taken up and used as substrate for steroid hormone production. The $^3$H products extracted from the media and isolated by TLC were not produced in the absence of cells and were prevented from accumulating by AG an inhibitor of cytochrome P-450 side chain cleavage, the committed step in steroid hormone synthesis. Similarly, inhibition of energy metabolism by cyanide/floride blocked both fluorogenic steroid and $^3$H-sterol production. Moreover, the labeled products extracted from the media with methylene chloride migrated coincident with or adjacent to unlabeled corticosterone and other steroid products in our TLC systems.

The major steroid products secreted by cultured rat adrenocortical cells include corticosterone, 18-hydroxycorticosterone, and deoxycorticosterone (54). We have previously reported (5) that these steroid products migrate to three poorly separate positions in the TLC system used. We have combined these spots in counting $^3$H-steroid products. Together they comprise the total cholesteryl ester undergoing side chain cleavage. Thus, the steroid products we have isolated provide a quantitative estimate of the amount of HDL cholesteryl esters converted to steroidaligenic products. The amount of steroidaligenic substrate derived from HDL cholesteryl esters is equivalent to the number of counts recovered as steroid products divided by the specific activity of added cholesteryl esters. When the amount of cholesteryl ester in HDL was not directly measured, we have assumed that it constituted 85% of the total HDL cholesterol (55, 56). To determine the percent of steroid product derived from HDL cholesteryl ester, it was also necessary to estimate the total quantity of steroid hormone produced. Fluorescent measurements do not directly measure total steroid production since not all steroid products yield an equivalent molar fluorescent signal. However, total steroid production can be estimated if the relative abundance of the products and their individual fluorescent intensities relative to the corticosterone standard are known. Of the major steroid products, only corticosterone fluoresces significantly (54). O’Hare and Neville (54), using radiolabeled pregnenolone, have shown that corticosterone constitutes approximately 50% of the total steroid production of cultured rat adrenal cells. Thus, fluorescence measurement, using corticosterone as a standard, underestimates total steroid mass by about 50%. The calculated contribution of cholesteryl esters to total steroid synthesis, based on these assumptions, varied from 24.5 to 100% and averaged 50 ± 18% (Table I). Thus, under the conditions of these experiments, the ester portion of HDL accounts for the majority of extracellular substrate.

In the experiments reported in Table I, total steroid output ranged from 0.42 to 2.6 μg/well/18 h. The average protein content/well was 66.6 ± 9.5 μg (triplicate measures from four preparations). Assuming the adrenal cortex is approximately 10% protein by weight, steroid production at an HDL concentration of 100 μg of cholesterol/ml, in our system would be approximately equivalent to 211 μg/g tissue/h. The reported circulating concentration of HDL cholesterol in the rat is approximately 300 μg/ml (57, 58). Thus, at circulating HDL concentrations, *in vitro* steroid production can be estimated to be 600–700 μg/g tissue/h. The initial rate of steroid production by prestimulated cells is perhaps twice as great as the rate at later times. However, this is considerably less than the maximal in *vivo* rate estimated by Spady and Dietschy (59) to be 4300 μg/g/h. The reasons for this difference are not known but could reflect the inherent uncertainties in the various estimates employed, differences in the degree of stimulation or responsiveness of tissues in *vivo* and *in vitro* and/or the amount of available cholesterol substrate. In *vivo* free cholesterol in HDL consumed by adrenal steroidogenesis can be constantly replenished at other sites in the circulation. In *vivo* the continuous replacement of consumed free cholesterol cannot occur. Thus, the contribution of free cholesterol is more limited *in vitro* than *in vivo*. 

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Over the course of an 18 h in vitro incubation using ACTH/LPP-pretreated cells, our results indicate that 70–100% of substrate is derived from HDL cholesteryl esters. These findings are consistent with and supportive of the in vitro observation of Spady and Dietschy (59) showing that endogenous cholesterol synthesis provides only 2–4% of substrate. Interestingly, the percentage of steroid products derived from cholesteryl esters consistently decreased as the concentration of HDL increased. Two explanations might explain this finding. First, increasing HDL concentration might stimulate endogeneous cholesterol synthesis (60). Alternatively, and we believe more likely based on our earlier inability to detect changes in cholesterol synthesis (5), unesterified cholesterol in HDL could provide an increasing percentage of substrate at higher HDL concentrations. In either event, at circulating concentrations, cholesteryl esters appear to provide the majority of substrate cholesteryl in vitro.

The current studies shed light on the mechanism and regulation of HDL cholesterol uptake. At least three mechanisms could explain adrenal uptake of cholesteryl ester from HDL. First, cholesteryl esters could be hydrolyzed and the resultant unesterified cholesterol taken up by a process of aqueous diffusion (3, 13). Our results indicate this is not the case. In the absence of cells, the rate of cholesteryl ester hydrolysis is much too slow to account for the observed uptake of [\(^{3}H\)]cholesteryl ester. Moreover, a substantial proportion of the accumulated [\(^{3}H\)]cholesterol was recovered in the ester form. If accumulation of HDL cholesteryl ester were mediated by hydrolysis and reesterification, then Sandoz 58-035, an inhibitor of acyl coenzyme A cholesterol acyltransferase, should have decreased cell [\(^{3}H\)]cholesteryl ester accumulation. It did not.

At least two observations indicate that the cellular accumulation of cholesteryl ester cannot be attributed to lipoproteins bound to the cell surface. First, we have previously shown that the majority of surface-bound HDL is rapidly released by repeated washing or by decreasing the extracellular concentration (30). Second, the amount of surface-bound HDL was directly estimated by measurement of cell-bound iodinated apoprotein. The ratio of cell accumulated [\(^{3}H\)]CE to bound [\(^{125}I\)]-apoprotein increased progressively and dramatically with time. Thus, the accumulation of [\(^{3}H\)]CE occurs in excess of bound lipoprotein.

Interestingly, in contrast to studies reported by Veldhuis et al. (61) in swine granulosa cells, inhibition of cholesterol esterification by Sandoz 58-035 did not enhance steroid hormone production. This difference in response could be due either to cellular differences in rates of unesterified cholesterol production from cholesteryl ester stores or to differences in the rate of free cholesterol transport or utilization. In any event, it appears that inhibition of esterification does not uniformly drive steroid hormone production in all species and steroidogenic glands. Finally, the inability of Sandoz 58-035 to alter rates of [\(^{3}H\)]steroid production from [\(^{3}H\)-CE HDL, indicates that accumulated HDL cholesteryl ester need not move through intracellular cholesteryl ester storage pools before undergoing side chain cleavage. The factors which influence the distribution of accumulated cholesteryl esters between storage and steroidogenic substrate pools are not yet known.

A second mechanism which could cause HDL cholesteryl ester uptake is receptor-mediated endocytosis (2). Three observations indicate that this is not the mechanism of uptake. First, the lipoproteins used in these studies were free of apoproteins which bind to currently recognized receptors. Second, the rate of HDL degradation, estimated by production of nontrichloroacetic acid precipitable iodinated apoprotein products, was insufficient to account for the amount of cholesteryl ester accumulated. Third, although specific HDL degradation was nearly completely inhibited by chloroquine, hydrolysis of HDL cholesteryl esters was not, indicating that hydrolysis of HDL cholesteryl esters does not occur intralysosomally (62). The lack of lysosomal cholesterol hydrolysis and the minimal amount of apoprotein degradation further indicates that uptake of HDL cholesteryl esters is not mediated by apoE secreted by the adrenal (63, 64) which then binds to added apoE-free HDL (65) and mediates uptake via the apoE receptor.

A third mechanism for HDL cholesteryl ester uptake, suggested by our earlier studies (5, 14) and those of Pittman and his colleagues (7) is specific cholesteryl ester membrane transport. Several properties of the uptake process are revealed by our studies. First, in ACTH-stimulated cells uptake progresses linearly with time for up to 26 h. Immediately following addition of [\(^{3}H\)]-CE-HDL to cells there is an initial rapid increase in cell-associated [\(^{3}H\)]-sters. The initial rapid uptake is similar in duration to the initial rate of [\(^{125}I\)]-HDL binding we have previously reported (30). However, even at the end of the 40–60-min period the ratio of cell accumulated [\(^{3}H\)]-sterol to cell-bound [\(^{125}I\)]-HDL exceeds the ratio in unincubated HDL indicating that selective uptake begins without delay in cells pretreated with ACTH. Subsequently, the rate of [\(^{3}H\)]-sterol accumulation continues linearly and greatly exceeds the rate at which cells accumulate [\(^{3}H\)]-apoprotein. Second, uptake of HDL cholesteryl ester is mediated by a saturable process as indicated by the finding that addition of excess unlabeled HDL inhibits not only production of [\(^{3}H\)]-steroids but also uptake per se. Further support for the saturable nature of the uptake process is provided by the HDL dose response curve (Fig. 10). Examination of the dependence of cholesteryl ester uptake and conversion to steroid hormones on HDL concentration suggests that these processes are comprised of at least two components, one of high and one of low affinity. It is not surprising that a complex dose response curve was observed since, undoubtedly, many steps occur between cell membrane HDL contact and cell cholesteryl ester accumulation and production of steroid products. We estimate the concentration of HDL required for half-maximal effect on the high affinity process to be approximately 16–20 \(\mu\)g of HDL cholesterol/ml. The current studies, which were conducted at relatively low HDL concentrations, permit only a rough estimate of the concentration of HDL required to half-maximally stimulate the low affinity process. By double reciprocal analysis, that concentration was estimated to be approximately 55 \(\mu\)g of HDL cholesterol/ml. This is consistent with our earlier studies showing that half-maximal HDL stimulation of steroid production and cholesterol accumulation occur at 230 and 210 \(\mu\)g of HDL-protein/ml. In the rat circulating HDL cholesterol concentration is approximately 300 \(\mu\)g/ml (67, 68), a concentration sufficient to fully saturate the high affinity process but one in which regulation of steroid output would still be dependent on circulating levels by virtue of the lower affinity process. The physical process(es) responsible for these complex HDL kinetics are not yet known but undoubtedly involve membrane-HDL binding as well as transmembrane and intracellular cholesteryl ester transfer and hydrolysis.
uptake was not inhibited by inhibition of steroidogenesis. Aminoglutethimide which is a competitive inhibitor of cholesterol side chain cleavage (66), effectively prevented synthesis of [3H]-steroids from [3H]-HDL cholesterol esters but also produced a compensatory equivalent increase in cell-associated [3H]cholesterol esters. Interestingly, addition of AG did not redirect [3H]cholesterol esters into a hydrolytic pathway. This is consistent with earlier findings of Mahaffee et al. (67) who have shown that addition of AG, while inhibiting side chain cleavage, does not prevent mitochondrial cholesterol accumulation. Freeman (68) has recently suggested that AG impedes movement of unesterified cholesterol from the plasma membrane to the mitochondrion. The apparent disparity between these two sets of observations could be explained either by cell-specific differences or by differences in handling of unesterified cholesterol and cholesterol esters.

Cholesterol esters taken up by rat adrenal cells from HDL can be disposed of in five ways. If hydrolyzed they can be (i) converted to steroid hormones or (ii) retained within the cell as unesterified cholesterol or (iii) released back into the media. If not hydrolyzed, they can be (iv) retained within the cell or (v) released into the media. The current studies do not allow us to determine the extent of resecretion of cholesterol esters. Exclusion of this potential pathway would not affect estimates of net cellular uptake of HDL cholesterol ester but would cause underestimation of total cell HDL cholesterol ester metabolism. The current studies demonstrate that rat adrenal cells, not surprisingly, hydrolyze HDL cholesterol esters. Two observations support this contention; these include direct measurements of [3H]-unesterified cholesterol production and the fact that HDL cholesterol esters can be converted to steroid production since cholesterol esters must first be hydrolyzed to undergo side chain cleavage. Two observations indicate that HDL cholesterol ester hydrolysis is not lysosomally mediated. First, although cholesterol ester metabolism increased linearly with time up to 24 h after the initial more rapid increase, there is no concomitant degradation to non-trichloroaetic acid susceptible products of the associated apoproteins indicating that the particle was not endocytosed intact. Second, the hydrolysis of HDL cholesterol esters is not inhibited by chloroquine which is known to inhibit lysosomal function. Rat adrenal cells possess at least two cholesterol ester hydrolases (69), an acid optimal and neutral ACTH-regulated enzyme. The failure of chloroquine to inhibit HDL cholesterol ester hydrolysis indicates that the lysosomal enzyme is not responsible for the observed hydrolysis.

Our findings suggest that the rate of cholesterol ester uptake exceeds the rate of hydrolysis since both [3H]-unesterified cholesterol and [3H]cholesterol esters accumulate with time when cells are presented with HDL [3H]cholesterol ester. The apparent difference in the rate of uptake and hydrolysis could be due to inherent properties of the responsible cholesterol esterase or to compartmentalization. Perhaps, cholesterol esters entering by high and low affinity uptake enter different pools, only one of which is subject to immediate hydrolysis. These findings may explain our earlier observation that addition of HDL produces not only increased steroid hormone production but also a concomitant increase in cell cholesterol content.

Interestingly, however, cell-associated iodinated apoprotein also accumulates steadily throughout this period (30). The relationship of apoprotein accumulation to cholesterol uptake and metabolism is unknown. The utilization of cholesterol esters in HDL consumed a considerable portion of added cholesterol esters (up to 17% of added counts, at low HDL concentrations). Pittman et al. (8, 70) have shown that selective uptake of HDL cholesterol ester is not confined to an unique HDL subtraction. The removal of such a large percentage of cholesterol ester from HDL would be expected to result in formation of redundant surface constituents. Perhaps this is the force which leads to slow but persistent cell accumulation of apoA-I in vitro observed in the current studies and previously reported (30). Such cell-mediated alterations in HDL structure could alter subsequent HDL function and catabolism, perhaps serving as a signal for HDL removal from the circulation.

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