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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 [^{3}H]CE-HDL to cells pretreated with adrenocorticotrophin in lipoprotein poor media resulted in a time- and concentration-dependent accumulation of [^{3}H]cholesteryl ester and production of [^{3}H] cholesterol and [^{3}H]corticosterone. HDL-CE metabolism could be described as the sum of a high affinity ([HDL-cholesterol]$_{½\ max}$ = 16 μg/ml) and low affinity ([HDL-cholesterol]$_{½\ max}$ > 70 μg/ml) process. [^{3}H]Cholesterol was found both intracellularly and in the media. Accumulation of [^{3}H]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 lysosomally mediated.

Aminoglutethimide prevented conversion of [^{3}H]CE-HDL to steroid hormones but did not inhibit [^{3}H]cholesteryl ester uptake. Cellular accumulation of [^{3}H] cholesteryl ester exceeded accumulation of ^{125}I-apoproteins 5-fold at 1 h and 35-fold at 24 h indicating selective uptake of cholesteryl ester moiety. 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[1] 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_3, 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 nondegradable 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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[1] The abbreviations used are: HDL, high density lipoprotein; ACTH, adrenocorticotrophin; 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.

of accumulated cholesteryl esters. Moreover, because the ethers cannot be metabolized they may have unappreciated pharmacologic effects intracellularly, although they are metabolized normally intravascularly (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 can selectively accumulate cholesteryl ethers and esters (6–8), only indirect evidence suggests that cholesteryl esters in HDL may be utilized by the adrenal for steroid substrate. 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 cholesterol can undergo side chain cleavage, the committed step in steroidogenesis, we have examined cell-mediated hydrolysis of HDL cholesteryl esters.

EXPERIMENTAL PROCEDURES

Lipoprotein Preparation, Labeling, and Characterization—To examine the metabolism of HDL cholesteryl esters independently of the apoB,E receptor pathway, we required HDL which was (*a*) free of apoE; (*b*) contained radiolabeled cholesteryl esters, (*c*) contained no radiolabeled unesterified cholesterol, and (*d*) retained native steroidogenic activity. Human HDL_3 was chosen since it is essentially free of apoE. In previous studies employing purified HDL_3 (14) not subjected to heparin-Sepharose chromatography, apoE was undetectable 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_3 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_3 further freed of apoE by passage over a heparin-Sepharose affinity column (25).

Human serum HDL was labeled with [^{3}H]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 0.15 M NaCl containing EDTA (0.01%). Our previous studies (15) 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 acyltransforase (27) by incubating 1.125B under nitrogen at 56 °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 [^{3}H] cholesteryl-oleate dried onto 100–200 mg of Celite 545 (Supelco Inc., Bellefonte, 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 μCi of [^{3}H]cholesteryl-oleate which had been purified by TLC. At the end of the labeling incubation, celite was removed by centrifugation and millipore (.02 micron) filtration. In preliminary experiments, 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 12800 to 75000 dpm/μg cholesterol 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, fluorogenic steroid production by ACTH/LPP-pretreated rat adrenal cells incubated 24 h with [^{3}H]cholesteryl oleate labeled or unlabeled 1.125B, added at equal total cholesterol concentration, was compared. In six comparisons of five different preparations, the mean steroid production for labeled materials (0.872 ± 0.338 μg/well/18 h) was identical to that for unlabeled 1.125B (0.887 ± 0.437 μg/well/18 h). Paired Student's *t* test showed that the difference between labeled and unlabeled materials was not different from zero ($n = 6$, $p = 0.837$) indicating that the labeling procedure had preserved native HDL activity. All labeled HDL_3 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 HDL_3 support steroidogenesis equally well. We have used 1.125B interchangeably with HDL_3. In some cases, HDL_3 was further purified 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 HDL_3 and human HDL were iodinated using a modification of the iodine monochloride procedure described by Bilheimer *et al.* (29) as previously described (30). Lipoprotein poor human or fetal calf serum were prepared by centrifugation at $d = 1.215$ g/ml for 48 h, as previously described (14). Unlabeled human HDL_3 ($1.125 < d < 1.215$ 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 modification of the method of O'Hare and Neville (31) as previously described (14). Cells were initially plated on multiwell dishes (Falcon 3847) in F-12/Dulbecco's modified Eagle's medium (1:1) supplemented with 10% fetal calf serum (Hyclone) plus penicillin (100 units/ml) and streptomycin (100 μg/ml) and gentamycin (100 μg/ml) plus 3% bovine brain growth supplement (standard media). The conditions of culture were similar to those described by McAllister and Hornsby (32). The bovine brain growth supplement was prepared by a modification of the method of Gospodarowicz *et al.* (33) omitting the final column purification. In some cases, 2% (v/v) Ultroser-G (IBF Biotechnics, Savage, MD) was substituted for bovine brain growth supplement. In preliminary experiments, addition of neither the bovine growth supplement nor Ultroser had any acute effect on steroidogenesis or metabolism of [^{3}H]cholesteryl-ester in HDL (data not shown).

Except where noted, we have used confluent monolayer cultures which were pretreated for 48 h with ACTH (10^{-7} 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 enzymatically using reagents supplied in kit form by Boehringer Mannheim (No. 124087, West Germany). Steroid hormone production was measured fluorometrically using a modification of the method of Kowal and Fiedler (34), 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 measured according to 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 chloroform/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 Products International, Mount Prospect, IL) scintillation mixture prior to counting using a Rackbeta liquid scintillation counter (LKB Instruments, Gaithersburg, MD). Cell cholesterol was extracted with isopropyl alcohol as described by Johnson *et al.* (13). Degradation of apoproteins in HDL was determined by measurement of total media ^{125}I 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.[2]

Materials—Heparin-Sepharose was obtained from Pharmacia LKB Biotechnology Inc. ^{125}I and [^{3}H](1,2)-cholesteryl-oleate were purchased from Du Pont-New England Nuclear. [^{3}H]Cholesteryl-oleate was chromatographed on TLC plates prior to use.

RESULTS

To determine if cholesteryl esters in HDL could serve as steroidogenic substrate, HDL_3 ($1.125 < d < 1.21$ g/ml) labeled with [^{3}H]cholesteryl-oleate was incubated with primary con-

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fluent monolayer cultures of rat adrenocortical cells pretreated with ACTH in lipoprotein poor media for 48 h. The following two types of labeled HDL preparations were used: (i) partially purified HDL_3 consisting of plasma of $d > 1.125$ g/ml (^{3}H-CE-1.125B) and (ii) fully purified HDL_3 prepared from 1.125B by centrifugation and freed of apoE by heparin-Sepharose chromatography (apoE-free ^{3}H-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 ^{3}H-CE-HDL (^{3}H-CE-1.125B) (Table I, experiments 1 and 2) and apoE-free-^{3}H-CE-HDL_3 (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 radiolabeled 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 ^{3}H-CE-HDL (*column 2*) increased sterol 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/fluoride (*column 5*) inhibited both fluorogenic and radiolabeled steroid production. Addition of a 5-fold excess of unlabeled HDL_3 (*column 6*) enhanced fluorogenic steriod production greater than that produced by addition of labeled HDL alone (*cf. column 2*) but inhibited conversion of ^{3}H-CE from HDL to steroid products.

Incubation of ^{3}H-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% *versus* 0.24 ± 0.03%, $p = .29$). In three similar cell-free incubations, from 0.94 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 ^{3}H-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 ^{3}H-CE-HDL with cells, ^{3}H-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 ^{3}H-CE from HDL hydrolyzed by cells incubated in the presence and absence of chloroquine. The amount of ^{3}H-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 ^{3}H-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

All incubations were conducted for 18 h in 15-mm miniwell cluster dishes in a total media volume of 0.4 ml. Cells were pretreated for 24 or 48 h with ACTH (10^{-7} M) in lipoprotein poor media. Steroid production in the absence of added lipoprotein ranged from 0.20 to 0.50 μg of corticosterone equivalent/well/18 h.

Experiment no.	[HDL-C]	Specific activity HDL-C	Sterol	% added dpm in sterol	Fluorogenic steroid due to HDL[a]	Estimated % product derived from HDL-CE[b]
	μg/ml	*dpm/μg*	*dpm/well*		*μg/well*	
HDL = 1.125B						
1	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.						
3	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	4,893	2.0	1.08	36.2
6	25	12,524	3,195	2.6	1.04	24.5
7	4.8	74,668	25,116	17.5	0.42	80
	9.6	74,678	48,609	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
8	12	12,487	5,806	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).

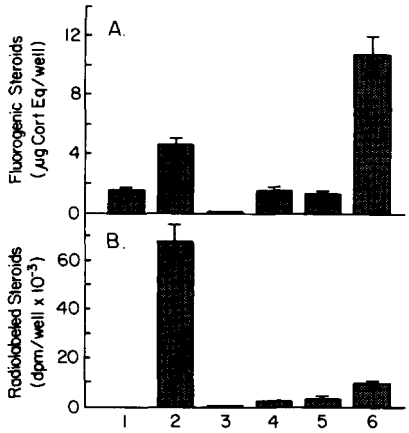

FIG. 1. **Effects of inhibiting steroidogenesis on conversion of ^{3}H-CE from HDL to steroid hormones.** Primary confluent monolayer cultures of rat adrenocortical cells, pretreated for 48 h with ACTH (10^{-7} M) in lipoprotein poor media, were incubated for 18 h with ACTH (10^{-7} M) (*all columns*) and H3-CE-1.125B (35 μg of cholesterol/ml) (*columns 2–6*) and the following additions: *column 4*, aminoglutethimide (800 μM); *column 5*, cyanide (10 mM) plus fluoride (10 mM); or *column 6*, unlabeled HDL (180 μg of cholesterol/ml). *Column 3* is identical with 2 except cell free. At the end of the incubation, the media was divided and fluorogenic steroid production was measured on 1 aliquot (*A*) and ^{3}H-steroids on the other (*B*). *Cort*, corticosterone equivalents.

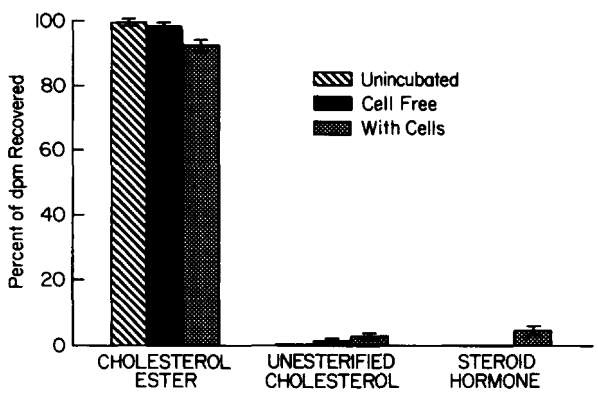

FIG. 2. **Cell-mediated hydrolysis of HDL cholesteryl esters.** ^{3}H-CE-1.125B (17.8 μg of total cholesterol/ml) was incubated for 24 h at 37 °C in 5% CO_2 in lipoprotein poor media, with confluent monolayer cultures of rat adrenocortical cells in 24-multiwell dishes. Control incubations were performed under identical conditions in the absence of cells. The resultant products were extracted, separated by TLC, and compared with similarly extracted and chromatographed unincubated ^{3}H-CE-1.125B held under nitrogen at 4 °C.

and specific receptor-mediated degradation of human ^{125}I-LDL by 81% (triplicate determination from triplicate experiments, data not shown).

To determine if cell-accumulated [^{3}H]cholesteryl esters arose from re-esterification of hydrolyzed HDL cholesteryl esters, we measured cell accumulation of ^{3}H-CE in the presence of Sandoz 58-035, an inhibitor of acyl-coenzyme A:cholesterol-acyltransferase (39). Preliminary studies were performed to determine the concentration of Sandoz 58-035 required to inhibit cholesterol esterification in rat adrenal cells. When cells were incubated with [^{3}H]cholesterol, added in ethanol, in the presence of increasing doses of Sandoz 58-035 and AG, to prevent conversion of accumulated [^{3}H]cholesterol to steroid products, dose-dependent inhibition of cholesterol esterification was observed (Fig. 4). Fifty % inhibition occurred at a concentration less than 0.1 μg/ml. To determine if inhibition of cholesterol esterification was due to generalized cell toxicity, we measured the effects of Sandoz 58-035 on steroidogenesis. When cells replete with endogenous cholesteryl ester stores were incubated with increasing doses of Sandoz 58-035, in the absence of AG, no effect on steroidogenesis was observed (Fig. 5*A*) even at concentrations of Sandoz 58-035 sufficient to almost completely inhibit cholesterol esterification.

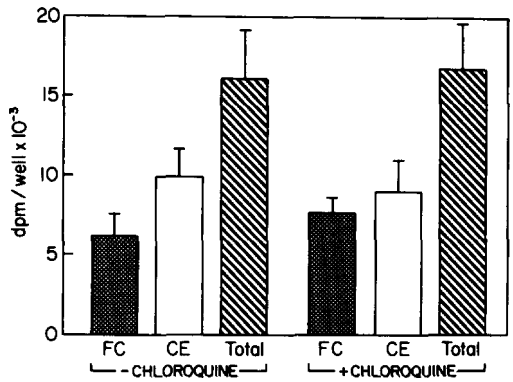

FIG. 3. **Effects of chloroquine on uptake and hydrolysis of HDL cholesteryl esters.** Primary confluent monolayer cultures of rat adrenocortical cells were incubated for 18 h at 37 °C with ^{3}H-CE-HDL (12.5 μg of total cholesterol/ml), AG (800 μM), and ACTH (10^{-7} M) in the presence and absence of chloroquine (100 μM). The cells were thoroughly washed and the cell-associated unesterified cholesterol and cholesteryl esters extracted and separated by TLC. Data shown are the results of triplicate determinations of duplicate experiments (mean ± S.D.). *FC*, free cholesterol; *CE*, cholesteryl ester.

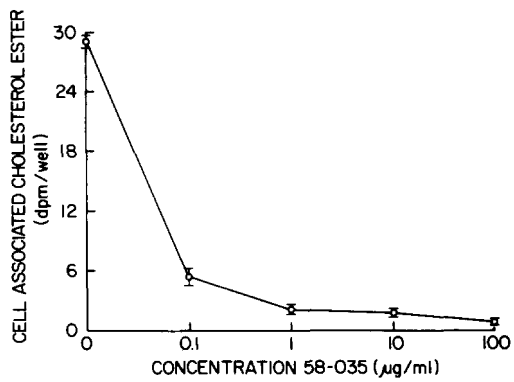

FIG. 4. **Dose-dependent inhibition of cholesterol esterification by Sandoz 58-035.** Primary monolayer cultures of rat adrenocortical cells were incubated for 18 h with [^{3}H]cholesterol (6.5 × 105 dpm) added in ethanol (final conc. = 2%) in the presence of increasing doses of Sandoz 58-035 added in 5 μl of Me_2SO and AG (800 μM) to prevent utilization of added cholesterol for steroidogenesis. Cell accumulated dpm were extracted with isopropyl alcohol and separated by TLC.

To determine if utilization of extracellular cholesterol required passage through the cholesteryl ester storage pool, we also measured the effects of Sandoz 58-035 on steroidogenesis in the presence of HDL. Cells were rendered dependent on extracellular lipoprotein cholesterol by pretreatment for 48 h with ACTH in lipoprotein poor media. Under these conditions, where 80% or more of the substrate cholesterol is derived from HDL (5, 14), Sandoz 58-035 had no effect on steroid hormone production (Fig. 5*B*) indicating that extracellular cholesterol need not pass through intracellular storage pools before being utilized for steroidogenic substrate.

Having shown that Sandoz 58-035 effectively inhibits cholesterol esterification without apparent cell toxicity we measured the effects of Sandoz 58-035 on cell accumulation of radiolabeled cholesteryl ester derived from HDL_3. Cells were

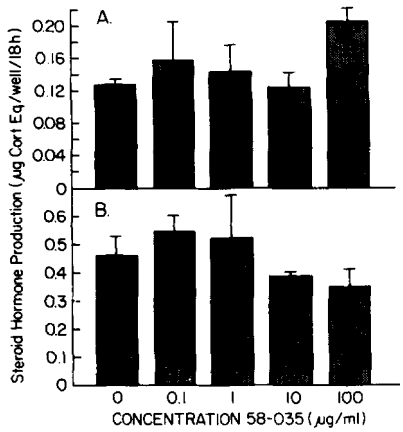

FIG. 5. **Effects of Sandoz 85-035 on HDL-dependent and independent steroidogenesis.** *A*, primary monolayer cultures of rat adrenocortical cells which had not been pretreated were incubated for 18 h in fresh complete media (V_{tot} = 0.5 ml) with increasing doses of Sandoz 58-035 (added in 0.005 ml of Me_2SO). Steroid production was measured fluorometrically. *B*, primary monolayer cultures of rat adrenocortical cells, pretreated for 72 h with ACTH/LPP (V_{tot} = 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 Me_2SO). Steroid production was measured fluorometrically (mean and S.D. of triplicate measures, typical of three such experiments). *Cort*, corticosterone equivalents.

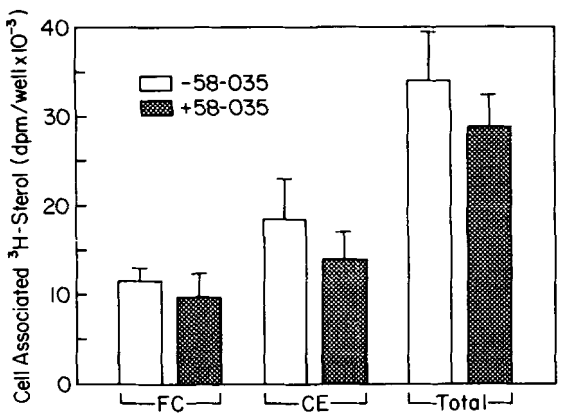

FIG. 6. **Effects of Sandoz 58-035 on cell accumulation of ^{3}H-cholesteryl ester from HDL.** Primary monolayer cultures of rat adrenocortical cells were pretreated for 48 h with ACTH/LPP and subsequently incubated for 18 h with ^{3}H-CE-1.125B (30 μg/ml total cholesterol, specific activity = 42,400 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.

incubated with ^{3}H-CE-1.125B in the presence of AG with and without Sandoz 58-035. In two separate experiments, addition of Sandoz 58-035 produced only a modest decrease in the total number of cell-associated counts and in the percent of cell-associated counts recovered as cholesteryl esters (Fig. 6). Despite effective inhibition of re-esterification, adrenal cells accumulate cholesteryl esters when incubated with ^{3}H-CE-HDL.

Having established that cholesteryl esters in HDL could be taken up by rat adrenal cells and used as steroidogenic substrate we next sought to further characterize the uptake process. To determine if cholesteryl ester uptake was secondary to steroid hormone synthesis or perhaps stimulated by extracellular steroid products, uptake was compared in the presence and absence of active steroidogenesis. When AG was added to ACTH/LPP-pretreated cells and incubated with ^{3}H-CE-HDL, cell-associated ^{3}H-sterols increased from 0.457 ± 0.141 to 0.576 ± 0.097 μg/well (n = 3, p = 0.042).

Net cellular metabolism of [^{3}H]cholesteryl ester from HDL is comprised of the sum of ^{3}H-steroid production, total cell-accumulated ^{3}H-CE and ^{3}H-unesterified cholesterol plus media ^{3}H-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 ^{3}H-CE from HDL to ^{3}H-steroids but did not diminish total cell metabolism (Fig. 7). In the absence of AG, addition of excess unlabeled HDL with ^{3}H-CE-HDL inhibited total cell metabolism of ^{3}H-CE (Fig. 7).

Cellular accumulations of ^{3}H-sterols from HDL was time (Figs. 8 and 9) and concentration (Fig. 10) dependent. When ^{3}H-CE-1.125B was added to ACTH/LPP-pretreated cells, there was an initial rapid increase followed by a prolonged

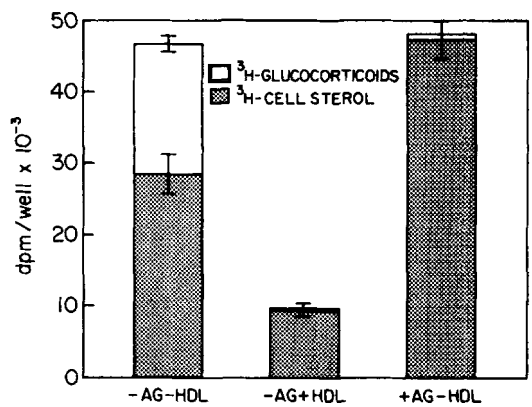

FIG. 7. **Effects of aminoglutethimide on uptake and metabolism of HDL cholesteryl esters.** Primary confluent monolayer cultures of rat adrenocortical cells, pretreated for 48 h with ACTH (10^{-7} M) in LPP media, were incubated for 18 h with ACTH (10^{-7} M), ^{3}H-CE-HDL (25 μg of total cholesterol/ml) with and without AG (0.8 mM) or unlabeled HDL (150 μg of total cholesterol/ml) as indicated. Total cell associated ^{3}H-cholesterol (FC + CE) were extracted from the cell pellet by isopropyl alcohol. Media ^{3}H-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.

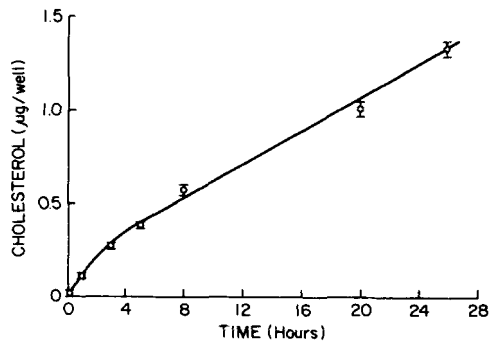

FIG. 8. **Time course of cellular accumulation of HDL cholesteryl esters.** Confluent monolayer cultures of rat adrenocortical cells, pretreated for 48 h with ACTH (10^{-7} M) in LPP media, were incubated for the indicated times with ^{3}H-CE-HDL (25 μg of total cholesterol/ml), ACTH (10^{-7} M), and AG (0.8 mM). The cells were thoroughly washed and cell associated [^{3}H]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.

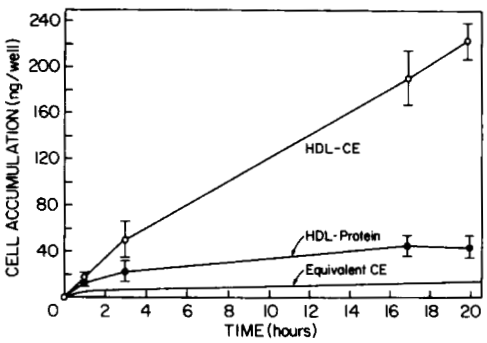

FIG. 9. **Comparison of the accumulation of [^{3}H]cholesterol and ^{125}I-protein from HDL by rat adrenocortical cells with time.** Primary confluent monolayer cultures of rat adrenocortical cells, pretreated for 48 h with ACTH (10^{-7} M) in LPP media, were incubated for the indicated times with ACTH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

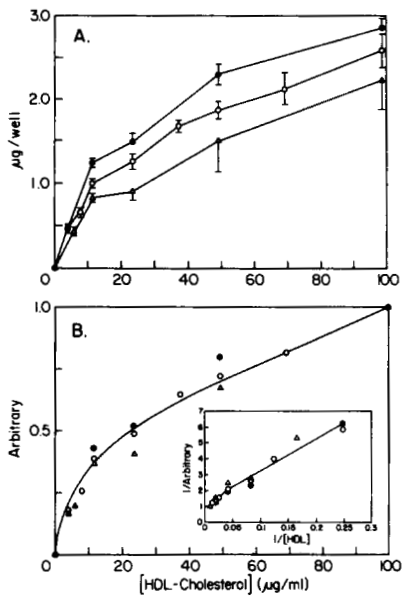

FIG. 10. **Concentration-dependence of fluorescent steroid production, ^{3}H-steroid production, and cell ^{3}H-sterol accumulation.** *A*, primary confluent monolayer cultures of rat adrenocortical cells, pretreated for 48 h with ACTH (10^{-7} M) in LPP media, were incubated for 18 h with ACTH (10^{-7} M) and increasing concentrations of ^{3}H-CE-HDL. Separate multiwell plates (24 × 15 mm) were used to measure total fluorogenic steroids (○) and ^{3}H-steroid (△) production in the absence of AG or ^{3}H-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 ^{3}H-sterol accumulation and ^{3}H-steroid production have been calculated from total dpm divided by the specific activity of cholesteryl ester in added HDL. *B*, 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.

linear increase over the ensuing 24 h (Fig. 8). The terminal rate of uptake of cholesteryl ester was 43 ng/well/h. To determine the extent to which cell-bound lipoprotein accounted for uptake of ^{3}H-CE, we compared cell accumulated cholesteryl ester with cell-metabolized ^{125}-I-apoHDL (Fig. 9). The total amount of ^{125}I-apoHDL metabolized by the cells theoretically includes both cell-associated material and degradation products. The latter were assessed as non-trichloroacetic acid precipitable ^{125}I in the media. When compared with cell-free controls, however, we could detect no difference in the amount of non-trichloroacetic acid precipitable ^{125}I in media from cells incubated with ^{125}I-HDL when compared with media from cell-free incubations. Thus, the data shown in Fig. 9 report only cell-associated ^{125}I. Moreover, the amount of cholesterol associated with degraded apoproteins was less than 10% of total metabolized cholesteryl ester. The rate of increase in cell-associated ^{3}H-sterols was much greater than the rate of increase in cell-associated ^{125}I. Moreover, the amount of cholesteryl ester taken up by the cells, based on measurements of accumulated ^{3}H-sterols, greatly exceeded the amount of cholesterol associated with the bound HDL estimated from measurements of cell-accumulated ^{125}I. The ratio of cell-associated ^{3}H-sterols to cell-associated ^{125}I-apoHDL increased with the duration of incubation. The ratio of cell-associated ^{3}H-sterols to cell-associated ^{125}I-HDL, when compared with the ^{3}H-^{125}I ratio in unincubated HDL, was 5-fold greater at 1 h and more than 35-fold greater at 24 h indicating selective metabolism of cholesteryl esters compared with apoHDL.

The effects of increasing HDL concentration on (i) fluorescent steroid production, (ii) production of ^{3}H-labeled steroid products, and (iii) cell-associated [^{3}H]cholesterol and cholesteryl esters was determined by incubating ^{3}H-CE-1.125B at increasing concentrations with ACTH/LPP-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 ^{3}H-steroids. All three parameters exhibited similar dependence on HDL concentration (Fig. 10*A*). 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. 10*B*). 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. 10*B*). 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 cholesterol 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 steroidogenic 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 steroidogenic substrate (14). Additional support for a role of cholesteryl esters arises from the studies of Glass *et al.* (5, 6) 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

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 intra- 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_3, which is free of apoB, 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 obviate 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/fluoride blocked both fluorogenic sterol 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 steroidogenic products. The amount of steroidogenic 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 217 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 vitro* the continuous replacement of consumed free cholesterol cannot occur. Thus, the contribution of free cholesterol is more limited *in vitro* than *in vivo*.

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 vivo* 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 cholesterol *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 apoB,E 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-sterols. 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 ^{125}I-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 μg 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 μg 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 μg of HDL-protein/ml. In the rat circulating HDL cholesterol concentration is approximately 300 μg/ml (57, 58), 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.

Third, the coincident dependent of fluorescent steroid production, cholesteryl ester accumulation, and production of ^{3}H steroids suggests that under the conditions employed in our studies, the rate of cholesteryl ester uptake determines the rate of subsequent processes. Finally, HDL cholesteryl ester

uptake was not inhibited by inhibition of steroidogenesis. Aminoglutethimide which is a competitive inhibitor of cholesterol side chain cleavage (66), effectively prevented synthesis of ^{3}H-steroids from ^{3}H-HDL cholesteryl esters but also produced a compensatory equivalent increase in cell-associated [^{3}H]cholesteryl esters. Interestingly, addition of AG did not redirect [^{3}H]cholesteryl 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 cholesteryl esters.

Cholesteryl 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 cholesteryl esters. Exclusion of this potential pathway would not affect estimates of net cellular uptake of HDL cholesteryl ester but would cause underestimation of total cell HDL cholesteryl ester metabolism. The current studies demonstrate that rat adrenal cells, not surprisingly, hydrolyze HDL cholesteryl esters. Two observations support this contention; these include direct measurements of ^{3}H-unesterified cholesterol production and the fact that HDL cholesteryl esters can be converted to steroid production since cholesteryl esters must first be hydrolyzed to undergo side chain cleavage. Two observations indicate that HDL cholesteryl ester hydrolysis is not lysosomally mediated. First, although cholesteryl ester metabolism increased linearly with time up to 24 h after the initial more rapid increase, there is no concomitant degradation to non-trichloroacetic acid susceptible products of the associated apoproteins indicating that the particle was not endocytosed intact. Second, the hydrolysis of HDL cholesteryl esters is not inhibited by chloroquine which is known to inhibit lysosomal function. Rat adrenal cells possess at least two cholesteryl ester hydrolases (69), an acid optimal and neutral ACTH-regulated enzyme. The failure of chloroquine to inhibit HDL cholesteryl ester hydrolysis indicates that the lysosomal enzyme is not responsible for the observed hydrolysis.

Our findings suggest that the rate of cholesteryl ester uptake exceeds the rate of hydrolysis since both ^{3}H-unesterified cholesterol and [^{3}H]cholesteryl esters accumulate with time when cells are presented with HDL [^{3}H]cholesteryl ester. The apparent difference in the rate of uptake and hydrolysis could be due to inherent properties of the responsible cholesteryl esterase or to compartmentalization. Perhaps, cholesteryl 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 cholesteryl esters in HDL consumed a considerable portion of added cholesteryl esters (up to 17% of added counts, at low HDL concentrations). Pittman *et al.* (8, 70) have shown that selective uptake of HDL cholesteryl ester is not confined to an unique HDL subfraction. The removal of such a large percentage of cholesteryl 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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