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Receptor-Mediated Uptake of Lipoprotein-Cholesterol and Its Utilization for Steroid Synthesis in the Adrenal Cortex

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I. Introduction

A. EVIDENCE THAT ADRENAL STEROIDS ARE DERIVED FROM PLASMA CHOLESTEROL

In 1945, Bloch fed isotopic cholesterol to a pregnant woman and isolated labeled pregenanediol from her urine, thus establishing that steroid hormones are derived from cholesterol (Bloch, 1945). In the succeeding 10 years, the major pathways for this conversion were elucidated, largely through studies of perfused bovine adrenal cortex. The latter studies were reviewed at a Laurentian Hormone Conference by Hechter *et al.* (1951). A major question then remained: How did the adrenal gland obtain the cholesterol that it used for steroid hormone synthesis?

In a classic experiment, Morris and Chaikoff (1959) fed [^{14}C]cholesterol to rats and found that the specific activity of adrenal cholesterol became equal to that of plasma. These data created the strong likelihood that the bulk of adrenal cholesterol in the rat was derived not from synthesis *in situ*, but rather by means of uptake from plasma cholesterol. Subsequently, Dexter, Fishman, and Ney (1970) showed that the uptake of [^{3}H]cholesterol from plasma into adrenal cortex was enhanced by adrenocorticotropin (ACTH) in rats. Finally, in a series of complex isotopics studies, Borkowski and co-workers (1970, 1972a,b) presented evidence that the bulk of adrenal cholesterol in humans is derived from plasma cholesterol.

Despite the overwhelming evidence that plasma cholesterol can gain access to the adrenal gland and the further suggestion that the uptake process might be regulated, few experiments were conducted to determine whether some type of specific uptake mechanism was involved. The possibility that such a specific uptake mechanism might exist was raised by the discovery in 1974 that cultured mammalian cells possess a cell surface receptor that facilitates the uptake of cholesterol carried in plasma lipoproteins (Brown and Goldstein, 1974; Goldstein

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and Brown, 1974). This lipoprotein uptake process was originally delineated in cultured human fibroblasts, which were shown to obtain all their cholesterol from the receptor-mediated uptake of one specific plasma lipoprotein, called low-density lipoprotein (LDL). In this paper, we briefly review the earlier studies of the LDL receptor in fibroblasts and then present the evidence that a similar receptor plays a key role in steroid synthesis in adrenocortical cells.

B. PLASMA LDL

Figure 1 shows a model of LDL, which is the major cholesterol-carrying lipoprotein in human plasma. The bulk of the cholesterol carried in LDL is located in an apolar core that contains approximately 1600 molecules of cholesterol per lipoprotein particle. Each molecule of cholesterol in this core is esterified with a long-chain fatty acid, the most abundant of which is linoleate. Surrounding this cholesteryl ester core is a polar coat consisting of phospholipids, relatively small amounts of free cholesterol, and a protein called apoprotein B (Jackson *et al.*, 1976; Kane, 1977). The structure of apoprotein B has not been elucidated, owing to its frustrating tendency to aggregate when it is delipidated. However, a growing body of evidence suggests that apoprotein B is composed of multiple identical subunits, each having a molecular weight of 25,000–35,000. Seven to ten of these subunits are associated to form a chain of approximately 240,000 daltons, and two such chains are present in each LDL particle. The nature of the chemical link between the apoprotein B monomers that form the two chains is not yet known (Bradley *et al.*, 1978; Deutsch *et al.*, 1978; Kuehl *et al.*, 1977).

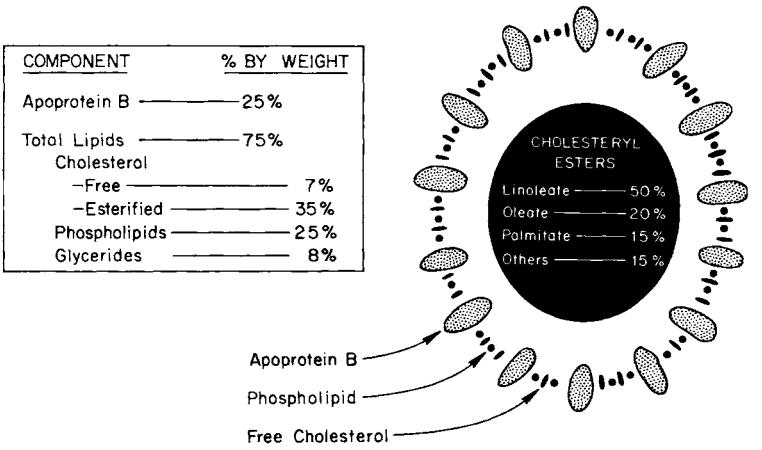

FIG. 1. Schematic diagram of the structure and composition of plasma low-density lipoprotein (LDL). Plasma LDL is depicted as a lipoprotein particle composed of an apolar core of esterified cholesterol that is surrounded by a polar coat composed of phospholipid, free cholesterol, and apoprotein B.

C. THE LDL RECEPTOR PATHWAY IN HUMAN FIBROBLASTS

Inasmuch as the bulk of the cholesterol in LDL is esterified, fibroblasts must have some way to hydrolyze these cholesteryl esters if they are to obtain the free cholesterol needed for membrane synthesis. The sequence of reactions by which LDL is taken up by the cells and its cholesteryl esters hydrolyzed has been called the LDL receptor pathway (Brown and Goldstein, 1976; Goldstein and Brown, 1976, 1977). As shown in Fig. 2, the critical component of this pathway is a cell surface receptor that specifically binds the apoprotein component of LDL (Brown and Goldstein, 1974; Goldstein *et al.*, 1976; Mahley *et al.*, 1977; Shireman *et al.*, 1977; Steinberg *et al.*, 1978). In fibroblasts these receptors are located in discrete segments of the plasma membrane called coated pits (Anderson *et al.*, 1976, 1977). Approximately once every 5 minutes, each coated pit invaginates into the cell and pinches off to form a coated vesicle that carries the receptor-bound LDL to lysosomes. Within the lysosomes, the protein component of LDL is hydrolyzed (Goldstein and Brown, 1974; Goldstein *et al.*, 1975a), the cholesteryl esters of LDL are cleaved by an acid lipase, and the resultant free cholesterol is transported from the lysosome into the cellular compartment (Brown *et al.*, 1975a,c; Goldstein, *et al.*, 1975b). In fibroblasts this free cholesterol is used primarily for membrane synthesis (Brown *et al.*, 1975c).

The cholesterol derived from LDL also serves as the mediating agent for three

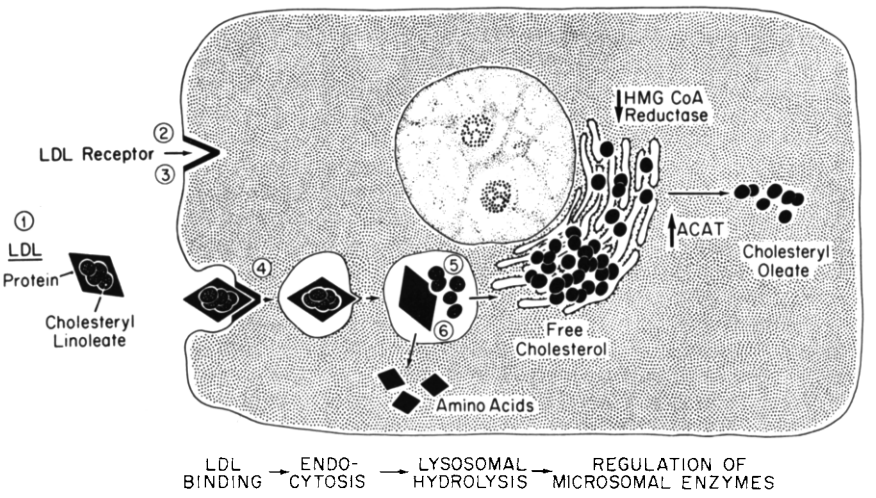

FIG. 2. Sequential steps in the low-density lipoprotein (LDL) receptor pathway. Numbers indicate the sites at which mutations have been identified in human cells: (1) abetalipoproteinemia; (2) familial hypercholesterolemia, receptor-negative; (3) familial hypercholesterolemia, receptor-defective; (4) familial hypercholesterolemia, internalization defect; (5) Wolman's disease; and (6) cholesteryl ester storage disease. HMG CoA reductase denotes 3-hydroxy-3-methylglutaryl coenzyme A reductase; ACAT denotes acyl-CoA: cholesterol acyltransferase. From Brown and Goldstein (1976).

regulatory actions that stabilize its intracellular concentration. First, the incoming cholesterol suppresses the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), the rate-controlling enzyme of cholesterol biosynthesis (Brown *et al.*, 1973, 1974). Second, the free cholesterol activates a microsomal acyl-coenzyme A:cholesteryl acyltransferase (ACAT), which esterifies any excess free cholesterol that is generated from LDL so that it can be stored as cholesteryl ester droplets (Goldstein *et al.*, 1974; Brown *et al.*, 1975b). Finally, the free cholesterol derived from LDL suppresses the synthesis of LDL receptors, thus preventing an overaccumulation of cholesterol (Brown and Goldstein, 1975).

An important concept arising from studies of the LDL pathway in human fibroblasts was that the receptor is specific for LDL; thus, under normal circumstances, LDL is the only lipoprotein that supplies cholesterol for cell growth. The other major cholesterol-carrying lipoprotein in human plasma is high-density lipoprotein (HDL), which contains two major proteins, apoproteins A-I and A-II. HDL does not bind to the LDL receptor and thus is not able to satisfy the cholesterol requirements of fibroblasts (Brown *et al.*, 1975c; Mahley *et al.*, 1977). In certain animals that are fed large amounts of cholesterol, a new lipoprotein, called HDL_c, accumulates in plasma (Mahley, 1978). Although this lipoprotein does not contain apoprotein B, it contains a protein with similar properties called the arginine-rich protein or apoprotein E. Because the arginine-rich protein can also bind to the LDL receptor, HDL_c can deliver cholesterol to fibroblasts just as can LDL (Bersot *et al.*, 1976; Mahley *et al.*, 1977; Mahley, 1978). HDL_c may be of importance in pathologic states; however, it has not been shown to occur in appreciable amounts in normal plasma, and hence its role in normal physiology is presently uncertain.

That the LDL receptor pathway is not limited to fibroblasts was established by the demonstration that LDL receptors were active in a variety of human cell types in culture, including arterial smooth muscle cells (Albers and Bierman, 1976; Brown *et al.*, 1977) and lymphoblasts (Ho *et al.*, 1976b). Moreover, this receptor pathway has been demonstrated in fibroblast lines from several other animal species, including mice, hamsters, swine, and monkeys (for review, see Goldstein and Brown, 1976, 1977). Finally, freshly isolated human lymphocytes have been shown to satisfy their cholesterol requirements by taking up LDL through the receptor pathway, suggesting that this receptor functions in cells in the human body (Ho *et al.*, 1976a, 1977; Bilheimer *et al.*, 1978).

The physiologic importance of the LDL receptor pathway became apparent when it was found that a series of allelic mutations in the gene specifying the LDL receptor causes familial hypercholesterolemia, one of the most common single gene-determined disorders affecting man (Brown and Goldstein, 1974, 1979). In heterozygotes with this disease, the number of functional receptors is reduced by one-half, the efficiency of degradation of LDL in the body is reduced by 50%, and the lipoprotein accumulates in plasma to levels of 2- to 3-fold above

normal, with the result that myocardial infarctions occur early in middle age. In patients with the homozygous form of this disease, LDL receptors are either absent or markedly reduced, the efficiency of LDL degradation is severely impaired, the lipoprotein accumulates to massive levels in plasma, and severe atherosclerosis occurs in the first few years of life (Fredrickson *et al.*, 1978; Goldstein and Brown, 1978) Other genetic defects in the LDL receptor pathway in man are also known (Fig. 2).

On the basis of the cited studies and experiments performed in other laboratories (for review, see Havel *et al.*, 1979), it has been suggested that LDL functions to transport cholesterol from absorptive and synthetic sites (intestine and liver, respectively) to extrahepatic parenchymal cells, which utilize the sterol for structural and metabolic purposes. In the absence of receptors or with a diminished number, LDL clearance from plasma is impaired and the atherogenic consequences of hypercholesterolemia become manifest.

II. The LDL Receptor Pathway in Adrenal Cortex

The elucidation of the LDL receptor pathway in fibroblasts immediately raised the possibility that a similar sequence might play a role in delivering cholesterol to steroid-secreting cells. In general, steroid-secreting cells require much more cholesterol than other cells since, in addition to the cholesterol needed for membrane synthesis, these cells require cholesterol for conversion to steroids. Accordingly, in 1975 we set out to look for the LDL receptor pathway in adrenal cells. Table I lists the four models systems that we have used to demonstrate its presence and physiological significance. In the remainder of this paper, we discuss each of these systems in detail. The data are then utilized to assemble a general working model for cholesterol homeostasis in the adrenal gland.

A. THE FIRST MODEL: MOUSE ADRENAL TUMOR CELLS IN CULTURE (Y-1 CLONE)

The first steroid-secreting cells that were demonstrated to possess an LDL receptor pathway were the Y-1 clone of functioning mouse adrenal tumor cells (Faust *et al.*, 1977). Early studies by Kowal (1970) had shown that these cells,

TABLE I

LDL Metabolism in Adrenal Cortex: Four Model Systems

1. *Mouse* adrenal tumor cells in culture (Y-1 clone)
2. *Rats* treated *in vivo* with 4-aminopyrazolopyrimidine (4-APP)
3. *Bovine* adrenal cortex
   a. Cells in culture
   b. Membranes prepared from *in vivo* tissues
4. *Human* fetal adrenal membranes

which were placed in culture by Sato and co-workers (Buonassisi *et al.*, 1962; Yasumura *et al.*, 1966), responded to ACTH by secreting 11β-hydroxydihydroprogesterone (11β-hydroxy-DHP) as the primary steroid. Their failure to produce corticosterone has been attributed to a loss of the 21-β-hydroxylase enzyme in tissue culture (Kowal, 1970). Figure 3 shows the amount of 11β-hydroxy-DHP secreted into the medium by the Y-1 cells when they were incubated in serum from which the lipoproteins had been removed (lipoprotein-deficient serum). When the cells were incubated in the absence of ACTH (Fig. 3A), little steroid was produced. The addition of human LDL caused only a slight enhancement. When the cells were incubated in the presence of ACTH, but still in the absence of lipoproteins, some increase in the output of 11β-hydroxy-DHP was observed (Fig. 3B). However, the response to ACTH was limited by the availability of lipoprotein cholesterol, as evidenced by the observation that the addition of human LDL produced a 3-fold increase in the amount of steroids secreted over 48 hours (Fig. 3B). The addition of human or mouse HDL did not stimulate steroid secretion. These data suggested that the mouse Y-1 adrenal cells might possess an LDL receptor that allows them to utilize the cholesterol of LDL for steroid synthesis (Faust *et al.*, 1977).

The presence of this receptor was demonstrated formally by incubating the Y-1 cells with ^{125}I-labeled LDL (^{125}I-LDL) at 4°C. As shown in Fig. 4, the adrenal cells bound the ^{125}I-LDL at a surface binding site that showed saturability and high affinity. When the cells were subsequently treated with heparin, most of the

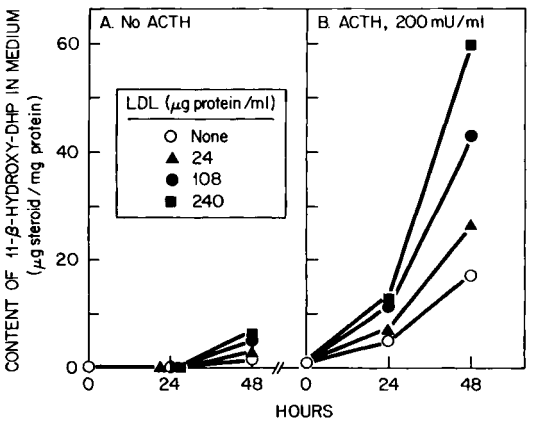

FIG. 3. Enhancement of steroid secretion by human low-density lipoprotein (LDL) in mouse Y-1 adrenal cells previously incubated in lipoprotein-deficient serum. On day 7 of cell growth, each monolayer received 2 ml of medium containing lipoprotein-deficient serum in the absence (A) or the presence (B) of 200 mU of ACTH per milliliter. On day 8 (zero time), the medium was replaced with fresh medium of the same composition but containing the indicated concentration of human LDL. After incubation at 37°C for an additional time as indicated, the medium was removed for measurement of its content of 11β-hydroxy-DHP and the cells were harvested for measurement of their total protein content. From Faust *et al.* (1977).

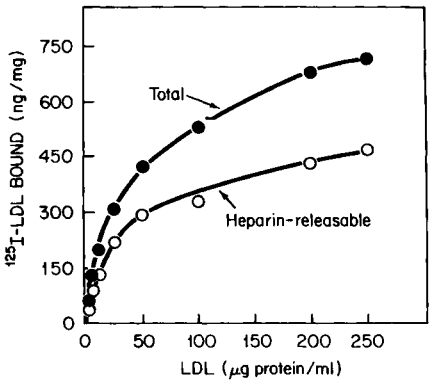

FIG. 4. Total and heparin-releasable binding of ^{125}I-labeled low-density lipoprotein (^{125}I-LDL) to mouse Y-1 adrenal cells at 4°C. On day 7 of cell growth, each monolayer received 2 ml of medium containing lipoprotein-deficient serum and 200 mU of ACTH per milliliter. On day 8, the cells were chilled to 4°C for 30 minutes, after which the indicated concentration of human ^{125}I-LDL (152 cpm/ng) was added to the medium. After incubation at 4°C for 2 hours, each monolayer was washed extensively, and the amount of total (●) and heparin-releasable (○) ^{125}I-LDL bound to the cells was determined. From Faust *et al.* (1977).

surface-bound ^{125}I-LDL was released. These findings are similar to those in human fibroblasts in which ^{125}I-LDL can be released from the receptor by exposure to sulfated gylcosaminoglycans, such as heparin and dextran sulfate (Goldstein *et al.*, 1976).

The lipoprotein receptor on the mouse adrenal cells was shown to bind both human and mouse LDL. On the other hand, neither human or mouse HDL competed for ^{125}I-LDL binding, indicating that the mouse adrenal receptor, like the human fibroblast receptor, was specific for LDL (Faust *et al.*, 1977). When the cells were incubated with ^{125}I-LDL continuously at 37°C, the receptor-bound LDL was internalized by the cell and the protein component was hydrolyzed in lysosomes, yielding [^{125}I]monoiodotyrosine, which was excreted into the culture medium. When the adrenal cells were incubated with ^{125}I-LDL in the presence of chloroquine, a nonspecific inhibitor of lysosomal enzymes (Goldstein *et al.*, 1975a), the hydrolysis of the lipoprotein was blocked, and intact ^{125}I-LDL accumulated progressively within lysosomes. Through the use of LDL whose cholesteryl ester component had been labeled with [^{3}H]cholesteryl linoleate, we showed that the cholesteryl ester component of LDL was also hydrolyzed within lysosomes (Faust *et al.*, 1977).

Uptake of LDL by the Y-1 cells regulated the processes of cholesterol synthesis and cholesterol esterification. When the cells were grown in the absence of lipoproteins, the activity of HMG-CoA reductase, the rate-controlling enzyme in cholesterol synthesis, was high. The resulting high rate of cholesterol synthesis within the cell was able to support cell growth. However, under these conditions, the cells did not accumulate any stored cholesteryl esters, and steroid secretion in

response to ACTH was minimal. When LDL was taken up through the receptor pathway, the free cholesterol released from the lysosomal hydrolysis of the lipoprotein satisfied the cholesterol requirement of the cells and suppressed HMG-CoA reductase (Faust *et al.*, 1977). The data in Fig. 5 show the degree of suppression of this enzyme that was obtained when the cells were incubated with either human or mouse LDL. On the other hand, mouse or human HDL, which did not bind to the LDL receptor, did not deliver cholesterol to cells or suppress HMG-CoA reductase activity.

The uptake of LDL-cholesterol by the Y-1 adrenal cells led to an enhancement in the rate at which the cells incorporated [^{14}C]oleate into cholesteryl [^{14}C]oleate. This enhancement was due to an increase in the activity of the microsomal cholesterol-esterifying enzyme ACAT (Faust *et al.*, 1977). As a result of this increased synthesis of cholesteryl esters, the Y-1 adrenal cells accumulated large amounts of stored cholesteryl esters in the presence of LDL (Fig. 6A). HDL caused no such effect. Similarly, LDL caused a marked stimulation in the rate of secretion of 11β-hydroxy-DHP, whereas HDL had no effect (Fig. 6B).

Figure 7 shows the time course of metabolism of LDL labeled with [^{3}H] cholesteryl linoleate after its addition to the monolayers of Y-1 adrenal cells. The left panel shows that in the absence of ACTH the content of [^{3}H]cholesteryl esters increased rapidly and reached a steady-state plateau. These esters were continually being hydrolyzed, generating free cholesterol, which also rose in the

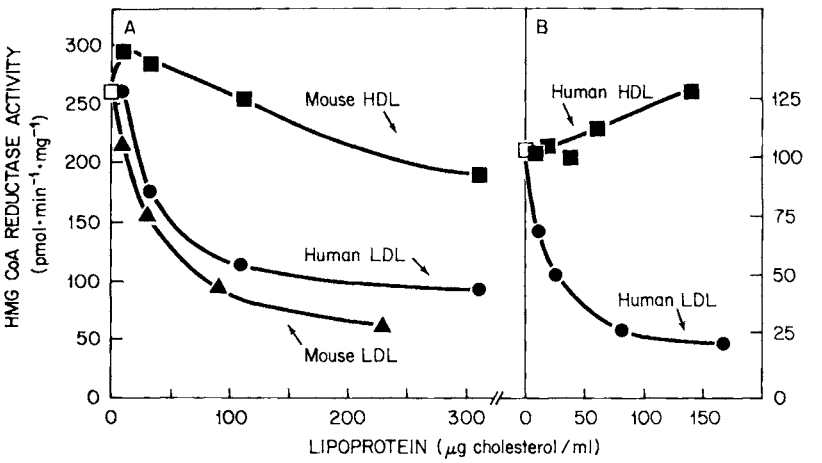

FIG. 5. Comparison of the ability of human and mouse low-density lipoprotein (LDL) and high-density lipoprotein (HDL) to suppress HMG CoA reductase activity in mouse Y-1 adrenal cells. On day 7, each monolayer received 2 ml of medium containing lipoprotein-deficient serum supplemented with the indicated concentration of one of the following lipoproteins. Experiment A: □, none; ●, human LDL; ▲, mouse LDL; or ■, mouse HDL. Experiment B: □, none; ●, human LDL; or ■, human HDL. After incubation for 24 hours at 37°C, the cells were harvested for measurement of HMG-CoA reductase activity. From Faust *et al.* (1977).

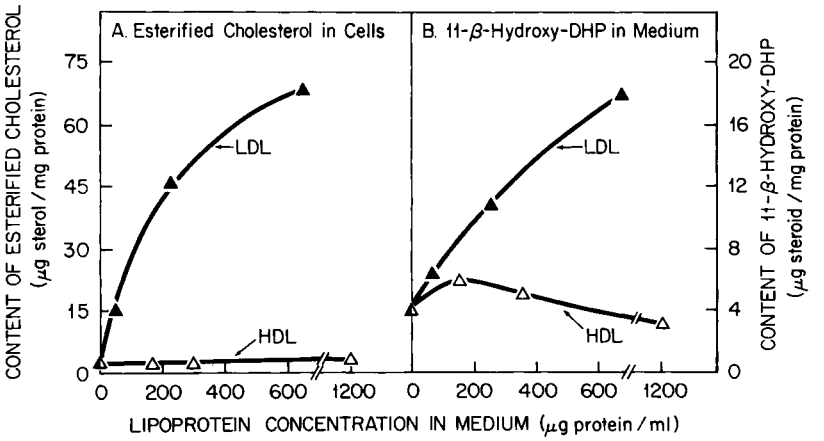

FIG. 6. Accumulation of esterified cholesterol (A) and stimulation of 11β-hydroxydihydroprogesterone secretion (B) in the presence of mouse low-density lipoprotein (LDL) but not high-density lipoprotein (HDL) in mouse adrenal Y-1 cells. On day 7, each monolayer received 2 ml of medium containing lipoprotein-deficient serum and 100 mU of ACTH per milliliter. On day 8, each monolayer received the same medium containing the indicated concentration of either mouse LDL or mouse HDL. After incubation for 24 hours at 37°C, the medium was removed for measurement of its content of 11β-hydroxy-DHP and the cells were harvested for measurement of their content of esterified cholesterol. From Faust *et al.* (1977).

cell until it too reached a steady state after about 24 hours (Fig. 7A). Even in the absence of exogenous ACTH, some of the free [^{3}H]cholesterol was converted to 11β-hydroxy-DHP (Fig. 7a, triangles). In the presence of ACTH the conversion of the LDL-derived [^{3}H]cholesterol to 11β-hydroxy-DHP was markedly accelerated (Fig. 7B, triangles). The kinetics suggest that [^{3}H]cholesterol liberated from the hydrolysis of LDL must first equilibrate with a cellular pool of [^{3}H] cholesterol before it is converted to steroids. We also showed that the addition of ACTH caused a 2- to 3-fold increase in the number of LDL receptors in the Y-1 cells, thus explaining the increased availability of cholesterol for steroid synthesis (see Fig. 16).

The experiments with the Y-1 cells demonstrated that, when these cells were grown in the steady state in the presence of LDL and when they were maximally stimulated with ACTH, approximately 75% of the secreted steriod was derived from the LDL-cholesterol obtained through the receptor-mediated pathway and 25% was derived from cholesterol synthesized within the cells (Faust *et al.*, 1977). At lower levels of steroid secretion, the proportion derived from LDL-cholesterol was even higher. In all its biochemical aspects, the LDL receptor of the Y-1 cells was similar to the LDL receptor in human fibroblasts. Moreover, the cholesterol derived from LDL regulated the same three events that it did in fibroblasts: suppression of HMG CoA reductase, stimulation of the ACAT, and suppression of the synthesis of LDL receptors.

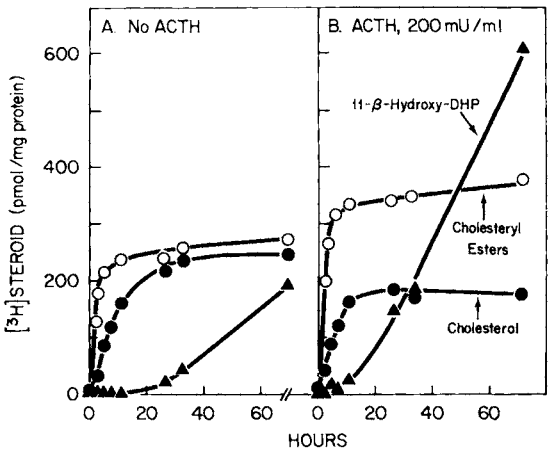

FIG. 7. Uptake and hydrolysis of [^{3}H]cholesteryl linoleate-labeled low-density lipoprotein (LDL) and secretion of 11β-hydroxy-[^{3}H]DHP in mouse adrenal Y-1 cells incubated in the absence (A) and in the presence (B) of ACTH. On day 7, each monolayer received 2 ml of medium containing lipoprotein-deficient serum in the absence (A) or in the presence (B) of 200 mU/ml of ACTH. On day 8 (zero time), the medium was replaced with fresh medium of the same composition but containing 1 μg of protein per milliliter of [^{3}H]cholesteryl linoleate-LDL (50,000 cpm/nmol of cholesteryl linoleate). After incubation at 37°C for the indicated time, the medium was removed and its content of 11β-hydroxy-[^{3}H]DHP (▲) was measured by thin-layer chromatography. The monolayers were than washed and harvested for measurement of the cellular content of [^{3}H]cholesteryl linoleate (○) and [^{3}H]cholesterol (●) by thin-layer chromatography. From Faust *et al.* (1977).

B. THE SECOND MODEL: RATS TREATED *in Vivo* WITH 4-AMINOPYRAZOLOPYRIMIDINE

To demonstrate that the adrenal gland *in vivo* was normally dependent upon lipoprotein-derived cholesterol for steroid hormone synthesis, we employed a rat model using the drug 4-aminopyrazolopyrimidine (4-APP) (Balasubramaniam *et al.*, 1976; Andersen and Dietschy, 1976). Henderson (1963) had demonstrated that this drug, which is an adenine analog, blocked the secretion of lipoproteins from liver in rats. Figure 8, taken from the work of Balasubramaniam *et al.* (1977b), shows that when rats were treated with 4-APP the plasma cholesterol level fell by more than 90% over the ensuing 24 hours. In association with the fall in plasma cholesterol levels, the cholesteryl ester content of the adrenal gland fell by more than 95% over 48 hours (Balasubramanian *et al.*, 1977b). In rats with normal plasma cholesterol levels, the rate of cholesterol synthesis in the adrenal is low (Dietschy and Wilson, 1970), and this was associated with a low activity of the rate controlling enzyme HMG-CoA reductase (Fig. 8B). However, when adrenal cholesteryl ester levels fell in the 4-APP-treated rat, the activity of HMG-CoA reductase rose by as much as 200-fold (Balasubramaniam *et al.*, 1977a,b), and cholesterol synthesis was increased by up to 50-fold (Andersen

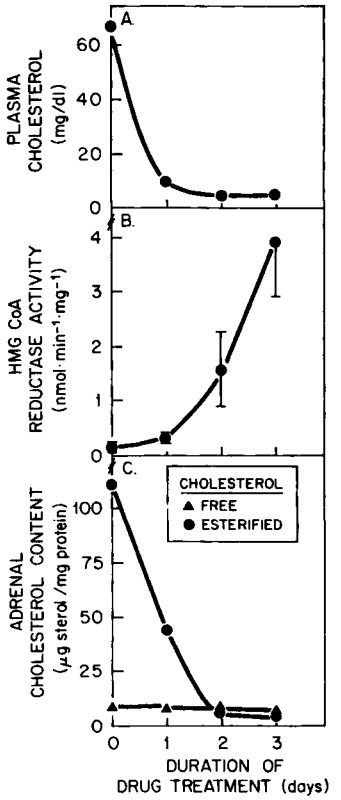

FIG. 8. Plasma cholesterol levels (A), adrenal HMG-CoA reductase activity (B), and adrenal cholesterol content (C) as a function of duration of administration of 4-aminopyrazolopyrimidine (4-APP) to rats. Daily doses of 4-APP (50 mg/kg) were administered intraperitoneally to rats for the number of days indicated. The rats were killed 24 hours after the last dose, and the indicated measurements were made. Each point represents the mean of values obtained from three rats. The brackets represent 1 SEM for the HMG-CoA reductase values. From Balasubramaniam *et al.* (1977b).

and Dietschy, 1976; Balasubramaniam *et al.*, 1977b). The fall in adrenal cholesteryl esters and the rise in HMG-CoA reductase was prevented when the animals were treated with dexamethasone, indicating that the massive stimulation of HMG-CoA reductase requires both hypocholesterolemia and high ACTH levels (Balasubramaniam *et al.*, 1977b).

Figure 9 shows that when the adrenal cholesteryl esters had been depleted by 4-APP treatment, the subsequent infusion of human LDL raised the plasma cholesterol level, replenished the adrenal cholesteryl esters, and suppressed HMG CoA reductase. As in the cultured mouse Y-1 adrenal cells, the accumulation of cholesteryl esters from LDL in the 4-APP-treated rat was associated with a marked enhancement in ACAT activity. Table II shows that in the 4-APP-

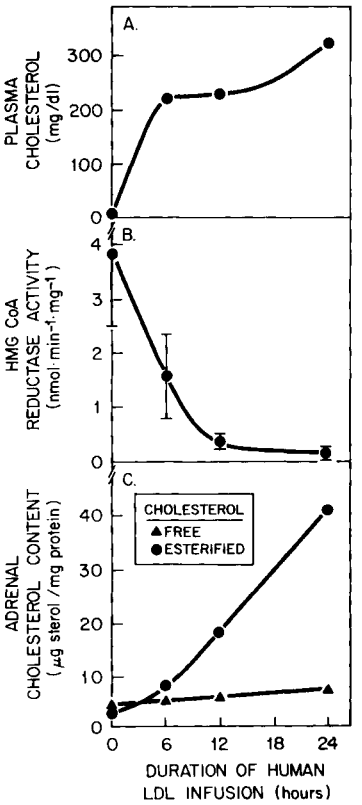

FIG. 9. Effect of human low-density lipoprotein (LDL) on the plasma cholesterol level (A), adrenal HMG-CoA teductase activity (B), and adrenal cholesterol content (C) of rats previously treated with 4-aminopyrazolopyrimidine (4-APP). Four groups of rats were treated intraperitoneally with 4-APP (50 mg/kg per day) for 3 days. The animals in three of these groups were then given an intravenous bolus injection of human LDL (20 mg of cholesterol), followed by a continuous infusion of the lipoprotein at the rate of 1.6 mg of cholesterol per hour for the indicated time. The control rats received no LDL. Each point represents the mean of values obtained from two or three rats. The brackets represent 1 SEM for the HMG-CoA reductase values. From Balasubramaniam *et al.* (1977b).

treated rat the ACAT activity of the adrenal gland decreased in parallel with the decrease in the esterified cholesterol content and the increase in HMG-CoA reductase activity. Subsequent infusion of LDL produced a 10-fold enhancement in the activity of the ACAT enzyme. The fatty acid composition of the cholesteryl esters that accumulated in the adrenal reflected this endogenous esterification activity. This follows from the observation that the cholesteryl esters of infused LDL were predominantly cholesteryl linoleate, while the esters that accumulated in the cell were predominantly cholesteryl oleate (Balasubramaniam *et al.*, 1977b). Thus, the increase in adrenal cholesteryl ester content did not

TABLE II

Reciprocal Changes in Activities of HMG-CoA Reductase and ACAT in Adrenal Gland of Rats Treated with 4-APP and Then Infused with Human LDL[a–c]

Treatment of rats	Plasma cholesterol level (mg/dl)	HMG-CoA reductase activity (nmol min^{-1} mg^{-1})	ACAT activity (pmol min^{-1} mg^{-1})	Content of esterified cholesterol (μg sterol/mg)
None	46	0.03	630	68
4-APP	3	6.40	65	5
4-APP + LDL infusion	128	0.05	678	35

[a] From Balasubramaniam *et al.* (1977b).

[b] Two groups of rats were treated intraperitoneally with 4-APP (50 mg/kg/day) for 3 days. The control rats received daily intraperitoneal injections of sodium phosphate buffer. Twelve hours after the third injection of 4-APP, each group of rats received an intravenous bolus injection either of 0.15 *M* NaCl or of human LDL (32 mg of cholesterol) as indicated. Twelve hours after the injections (on day 4) all animals were killed. Each value represents the mean ± 1 SEM of values obtained from three rats.

[c] HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; ACAT, acyl-CoA:cholesteryl acyl-transferase; 4-APP, 4-aminopyrazolopyrimidine; LDL, low-density lipoprotein.

result simply from the uptake of intact LDL, but rather arose from the reesterification of free cholesterol derived from the uptake and lysosomal hydrolysis of the cholesteryl esters of LDL.

In a subsequent study it was shown that the regulation of cholesterol synthesis in the adrenal of the 4-APP-treated rat resulted not only from changes in HMG-CoA reductase activity, but also reflected changes in 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMG-CoA synthase), the enzyme immediately preceding HMG-CoA reductase in the cholesterol biosynthetic pathway (Balasubramaniam *et al.*, 1977a). The data in Table III demonstrate the parallel increase in HMG-CoA synthase and HMG-CoA reductase in the 4-APP-treated rat. In contrast, acetoacetyl-CoA thiolase and mevalonate kinase, the enzymes immediately preceding and following the synthase and reductase in the cholesterol synthetic pathway, did not change significantly. The data in Fig. 10 also demonstrate that HMG-CoA synthase and reductase were suppressed in parallel when the cholesterol content of the plasma was restored by infusion of human LDL into the 4-APP-treated rats. Once again, thiolase and kinase activities did not change.

An interesting by-product of these studies was the observation that adrenal HMG-CoA reductase and HMG-CoA synthase normally undergo a diurnal rhythm. As shown in Fig. 11, the high point of the cycle was seen at about 1:00 AM, the midpoint of the dark phase. The peak occurred several hours after the peak in plasma corticosterone levels (Balasubramaniam *et al.*, 1977a), suggesting that cholesterol synthesis was activated after the gland had secreted large amounts of steroid.

In related studies, we showed that 3 hours after the infusion of ACTH into otherwise untreated rats the cholesteryl ester content of the adrenal gland dropped by about 50% and HMG-CoA reductase activity rose. However, in these intact animals, which had normal plasma lipoprotein levels, the continuation of the ACTH infusion led to a rise in the level of adrenal cholesteryl esters to control levels by 6 hours and to a fall in HMG-CoA reductase activity during the same interval (Balasubramaniam *et al.*, 1977b).

Taken together, the above experiments demonstrate that the adrenal gland of the rat responds to ACTH immediately by hydrolyzing its stored cholesteryl esters and transiently activating cholesterol synthesis. However, when plasma cholesterol is available, the uptake of plasma lipoprotein cholesterol soon takes place, and the gland returns to a new steady state with low HMG-CoA reductase levels and high cholesteryl ester stores. When plasma cholesterol is not available owing to 4-APP treatment, cholesteryl ester levels in the gland continue to fall, and cholesterol synthesis continues to rise.

The general pattern of these results was similar to the pattern of regulation that had been demonstrated in the cultured mouse Y-1 cells. However, one important difference was noted. Whereas in the Y-1 cells the cholesterol requirement was satisfied by a lipoprotein receptor that specifically bound LDL and did not

TABLE III

Effect of 4-APP on Enzymes of the Cholesterol Biosynthetic Pathway in Rat Adrenal Gland[a–c]

Treatment of rats	Cytosolic acetoacetyl-CoA thiolase (nmol min^{-1} mg^{-1})	Cytosolic HMG-CoA synthase (nmol min^{-1} mg^{-1})	Microsomal HMG-CoA reductase (nmol min^{-1} mg^{-1})	Cytosolic mevalonate kinase (nmol min^{-1} mg^{-1})
(a) Control	310	0.29	0.12	3.3
(b) 4-APP	240	4.1	6.1	2.3
(b)/(a)	0.8	14	51	0.7

[a] From Balasubramaniam *et al.* (1977a).

[b] Rats were treated as indicated, adrenal glands from four rats were pooled, cytosolic and microsomal fractions were prepared, and enzyme activities were determined as previously described.

[c] 4-APP, 4-aminopyrazolopyrimidine; HMC-CoA, 3-hydroxy-3-methylglutaryl coenzyme A.

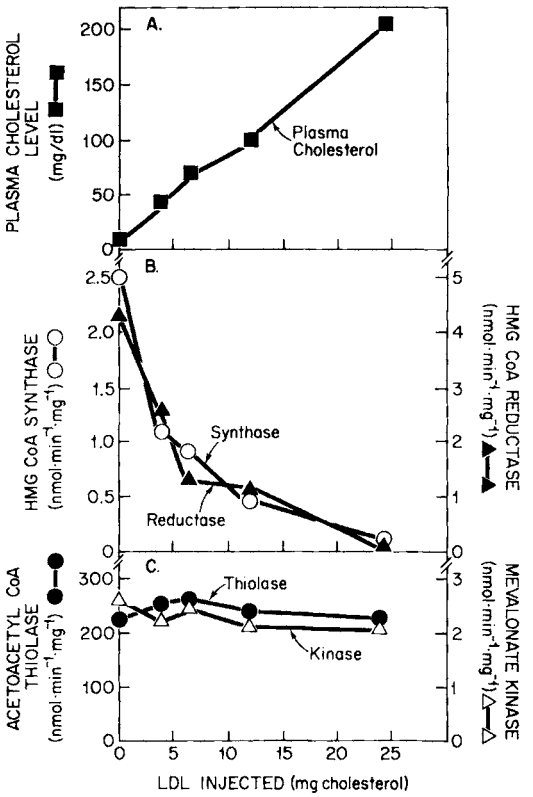

FIG. 10. Effect of various doses of human low-density lipoprotein (LDL) on the plasma cholesterol level (A), adrenal HMG CoA synthase and reductase activities (B), and adrenal acetoacetyl-CoA thiolase and mevalonate kinase activities (C) of rats previously treated with 4-aminopyrazolopyrimidine (4-APP). Five groups of rats (three rats per group) were treated with 4-APP for 3 days. Twelve hours after the last dose of 4-APP, four groups of rats received the indicated amount of human LDL intravenously as a bolus. The control group received an intravenous injection of 0.15 *M* NaCl. All the animals were killed 12 hours later (on day 4). From Balasubramaniam *et al.* (1977b).

recognize HDL, in the intact rat HDL was several times more effective than LDL in supplying cholesterol to the adrenal gland (Balasubramaniam *et al.*, 1977b). The data in Fig. 12 show that administration of human HDL to the 4-APP-treated rat produced an increase in the cholesteryl ester content of the adrenal gland and suppressed HMG-CoA reductase activity. Similar results were obtained using rat HDL.

Andersen and Dietschy (1976, 1977) have also observed that both HDL and LDL can deliver cholesterol to the adrenal gland of the 4-APP-treated rat. In careful quantitative comparisons, these authors showed that, whereas the maximal rate of uptake of cholesterol was similar from HDL and LDL, the concentra-

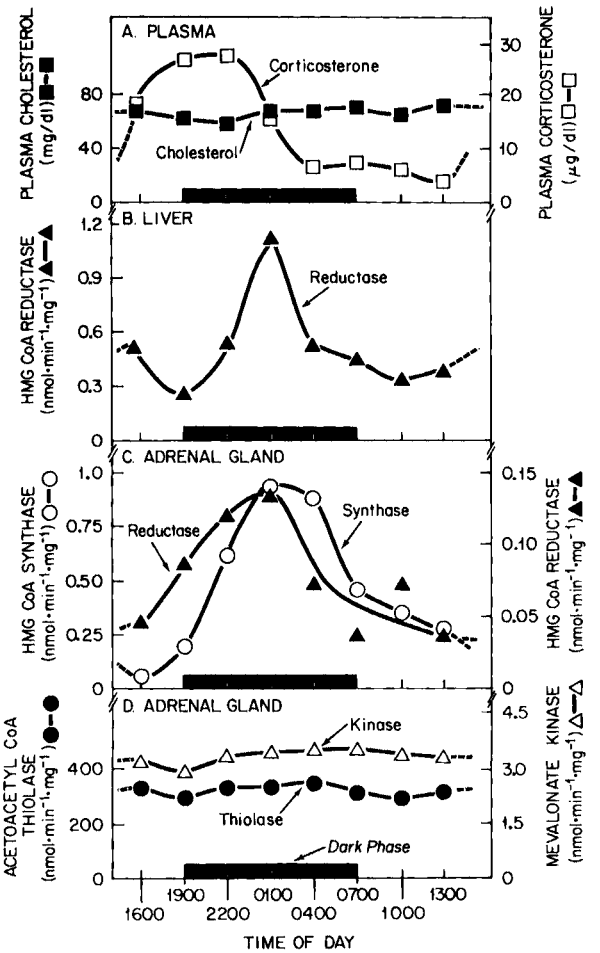

FIG. 11. Diurnal variations in the level of plasma corticosterone (A) and in the activities of hepatic HMG-CoA reductase (B), adrenal HMG-CoA synthase and reductase (C), and adrenal acetoacetyl-CoA thiolase and mevalonate kinase (D) in the rat. Twenty-four rats were housed in cages in which lighting was supplied between the hours of 0700 and 1900 daily. After 3 weeks of such treatment, groups of three animals were killed at intervals of 3 hours as indicated. Blood was collected, and the livers and adrenal glands from the three animals were excised and pooled for homogenization. From Balasubramaniam *et al.* (1977a).

tion of HDL-cholesterol required for half-maximal uptake was 4-fold less than that required for LDL-cholesterol (Dietschy, 1978).

At least four explanations can be suggested to account for the rat data: (1) the rat adrenal gland possesses a receptor that can somehow bind both human HDL and LDL, even though these two lipoproteins share no proteins in common; (2) the rat adrenal gland possesses a receptor that is specific for a distinct lipoprotein that can be derived from both human HDL and LDL during circulation in the

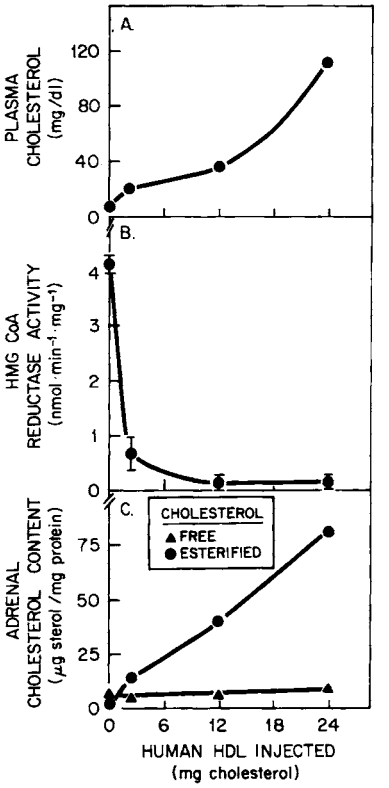

FIG. 12. Effect of human high-density lipoprotein (HDL) on the plasma cholesterol level (A), adrenal HMG-CoA reductase activity (B), and adrenal cholesterol content (C) of rats previously treated with 4-aminopyrazolopyrimidine (4-APP). Four groups of rats were treated intraperitoneally with 4-APP (50 mg/kg per day) for 3 days. Twelve hours after the last dose of 4-APP, three groups of rats received the indicated amount of human HDL intravenously as a bolus. The control group received no HDL. All the animals were killed 12 hours later (on day 4). Each point represents the average of values obtained from two or three rats. The brackets represent 1 SEM for HMG-CoA reductase values. From Balasubramaniam *et al.* (1977b).

4-APP-treated rat; (3) the rat adrenal gland possesses two distinct receptors, one for HDL and one for LDL; or (4) no specific receptors analogous to the mouse Y-1 adrenal cells are involved and the rat adrenal takes up cholesterol by a mechanism that shows no lipoprotein specificity. At present it is not possible to decide among these four possibilities. Gwynne and co-workers (1976) showed that radiolabeled free cholesterol was taken up more rapidly from HDL than from LDL in rat adrenal slices, but unidirectional flux was not measured and the uptake of labeled cholesterol may actually have represented isotope exchange.

It should be pointed out that the rat has the lowest plasma level of LDL of any mammalian species. In contrast to the human, where the plasma LDL-cholesterol

level is 2- to 3-fold higher than the HDL-cholesterol level, in the rat the HDL-cholesterol level is 2- to 3-fold higher than the LDL-cholesterol level (Havel *et al.*, 1979). Because of this deficiency of LDL, it might be expected that the rat uses some mechanism in addition to the LDL receptor to supply large amounts of cholesterol to the adrenal gland. A different situation seems to exist in larger animals including the cow, swine, dog, and man. In these species the concentration of plasma LDL is much higher than in the rat, and in each of these species a specific high-affinity LDL binding site can be demonstrated on isolated adrenal membranes (discussed below).

C. THE THIRD MODEL: BOVINE ADRENAL CORTEX

1. Adrenocortical Cells in Culture

The studies of the Y-1 cells of the mouse and the adrenal gland of the 4-APP-treated rat established the principle that plasma lipoprotein cholesterol could be used as the primary source of adrenal cholesterol for conversion to steroid hormones. However, as discussed above, the data suggested that different mechanisms of lipoprotein uptake might be involved in the two systems. The cultured mouse Y-1 cells used a specific receptor that only recognized LDL, whereas the rat adrenal gland *in vivo* appeared able to use cholesterol that was infused intravenously either in the form of LDL or HDL. To determine whether this difference was related to a species difference or to a difference in the behavior of cultured cells versus cells in the body, we recently turned to a system that allows a direct comparison of *in vivo* and *in vitro* results. These studies were made possible by the recent work of Gospodarowicz *et al.* (1977) and Hornsby and Gill (1977, 1978), who have developed a method to establish primary cultures of functioning adult bovine adrenocortical cells. Cells are dissociated from the adrenal cortex by digestion with collagenase and are then plated in Petri dishes. When grown in the presence of fibroblast growth factor, the cells divide and form confluent monolayers. They can be subcultured for up to 50 generations, and they respond to ACTH, cholera toxin, or prostaglandins by secreting steroids into the culture medium (Hornsby and Gill, 1977, 1978). However, since the cells rapidly lose the 11β-hydroxylase enzyme, they produce a variety of 11-deoxysteroids instead of cortisol or corticosterone (Simonian *et al.*, 1978).

Hornsby and Gill kindly sent us a vial of primary bovine adrenocortical cells, and we were able to maintain the line in cell culture (Kovanen *et al.*, 1979b). The data in Fig. 13A demonstrate that when the cells were grown in the absence of lipoproteins and in the absence of a stimulus to steroid secretion, only small amounts of fluorogenic steroids were secreted. The addition of LDL or HDL under these conditions did not stimulate steroid secretion significantly. When the cells were grown in the presence of cholera toxin, steroid output remained low in the absence of lipoproteins (Fig. 13B). However, under identical conditions the

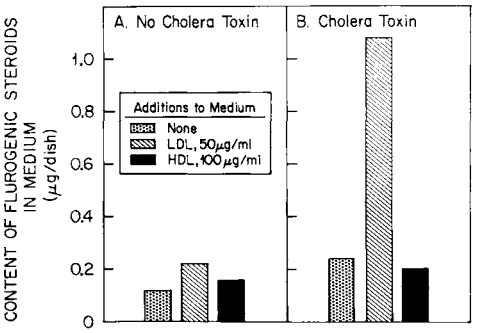

FIG. 13. Fluorogenic steroid secretion by monolayers of bovine adrenocortical cells after addition of cholera toxin and human lipoproteins to the culture medium. On day 6 of cell growth, each monolayer received 2 ml of medium containing lipoprotein-deficient serum and either no cholera toxin (A) or 0.8 μg of cholera toxin per milliliter (B). On day 7 (zero time), the medium was replaced with the identical medium supplemented with one of the following human lipoproteins: none, 50 μg of protein per milliliter of low-density lipoprotein (LDL) or 100 μg of protein per milliliter of high-density lipoprotein (HDL). Every 24 hours the medium was replaced with fresh medium containing the indicated addition. On day 10 the medium was removed for measurement of fluorogenic steroids that had accumulated during the 24-hour interval between days 9 and 10. The average content of cell protein in each dish at the end of the experiment was 300 μg. From Kovanen *et al.* (1979b).

addition of LDL produced a 5-fold stimulation of fluorogenic steroid secretion. The addition of HDL produced no such effect. Similar results were obtained with either bovine or human lipoproteins.

The data in Fig. 14 further illustrate the dependence of the bovine adrenocortical cells on the presence of LDL in order to achieve maximal steroid output. When the cells were incubated with increasing amounts of ACTH, there was little stimulation of steroid secretion unless LDL was present in the medium (Fig. 14A). Similar results were obtained with cholera toxin (Fig. 14B). These data suggested that the bovine adrenocortical cells in culture possessed a specific LDL receptor that was similar to the one previously described for human fibroblasts and cultured mouse Y-1 adrenal cells.

To demonstrate this receptor directly, we incubated the bovine adrenocortical cells with increasing amounts of ^{125}I-LDL and ^{125}I-HDL. The data in Fig. 15A show that as the concentration of ^{125}I-LDL was increased in the culture medium the cellular content of radioactivity rose with saturation kinetics. No high-affinity uptake process of ^{125}I-HDL could be demonstrated. Similarly, the cells degraded the ^{125}I-LDL by a saturable high-affinity process (Fig. 15B). No high-affinity degradation of either human or bovine ^{125}I-HDL could be demonstrated.

Additional experiments showed that human or bovine LDL, but not human or bovine HDL, suppressed HMG-CoA reductase activity, stimulated cholesteryl ester formation, and increased the cholesteryl ester content of the bovine adrenocortical cells (Kovanen *et al.*, 1979b). These results were identical to those previously obtained with the cultured mouse Y-1 Cells (Faust *et al.*, 1977).

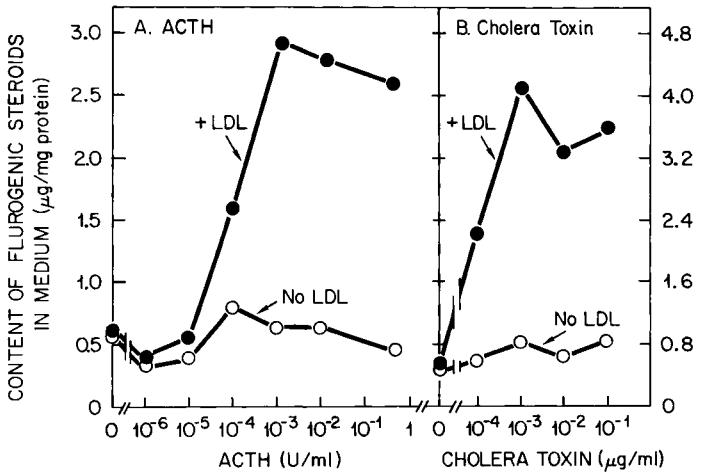

FIG. 14. Stimulation of fluorogenic steroid secretion in monolayers of bovine adrenocortical cells by varying concentrations of ACTH (A) and cholera toxin (B) in the absence (○) and in the presence (●) of human low-density lipoprotein (LDL). On day 6 of cell growth, each monolayer received 2 ml of medium containing lipoprotein-deficient serum and the indicated concentration of either ACTH or cholera toxin. On days 7 and 8 the medium was replaced with the identical medium supplemented with no LDL (○) or 50 μg of protein per milliliter of LDL (●). On day 9, the medium was removed for measurement of the amount of fluorogenic steroids that had accumulated during the 24-hour interval between days 8 and 9. From Kovanen *et al.* (1979b).

Conversion of LDL-cholesterol to steroids was demonstrated directly with the use of LDL in which the endogenous cholesteryl esters had been replaced with exogenous [^{3}H]cholesteryl linoleate (Krieger *et al.*, 1978). When this reconstituted lipoprotein was incubated with the bovine adrenocortical cells in the presence of cholera toxin, the cholesteryl esters of LDL were rapidly converted to ^{3}H-labeled cholesterol and secreted steroids. The major ^{3}H-labeled secreted steroid that was detected was 11-deoxycortisol (Kovanen *et al.*, 1979b).

In both the bovine adrenocortical cells and the mouse Y-1 adrenal cells, the activity of the LDL receptor was susceptible to metabolic and hormonal regulation. The data in Fig. 16A show that when bovine adrenocortical cells were incubated in the absence of LDL the activity of the LDL receptor was relatively high and the activity could be stimulated an additional 3-fold by the addition of cholera toxin. When the cells were grown in the presence of LDL and in the absence of a stimulus to hormone secretion, LDL receptor activity was quite low. Under these conditions, the addition of cholera toxin also produced an increase in LDL receptor activity. We calculated that in the presence of LDL the amount of LDL-cholesterol supplied by this increase in LDL receptor activity could account for all of the measured increase in steroid production in the presence of cholera toxin in the bovine adrenocortical cells. Similar results were obtained with ACTH (Kovanen *et al.*, 1979b). The data in Fig. 16B show that ACTH also

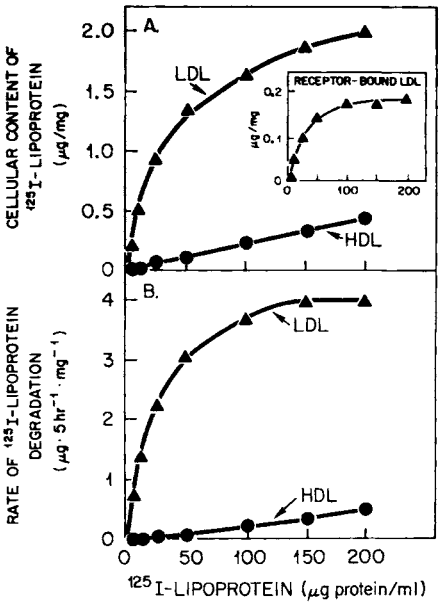

FIG. 15. Saturation curves for total uptake (A) and degradation (B) of human ^{125}I-labeled low-density lipoprotein (^{125}I-LDL) (▲) and human ^{125}I-labeled high-density lipoprotein (^{125}I-HDL) (●) in monolayers of bovine adrenocortical cells. On day 6 of cell growth, each monolayer received 2 ml of medium containing lipoprotein-deficient serum and 0.4 μg/ml of cholera toxin. On day 8 the medium was replaced with the identical medium supplemented with the indicated concentration of either ^{125}I-LDL (87 cpm/ng) or ^{125}I-HDL (64 cpm/ng). After incubation for 5 hours at 37°C, the total cellular content of ^{125}I-labeled lipoprotein (A) and total amount of ^{125}I-labeled lipoprotein degraded (B) were determined. The inset in panel A shows the amount of ^{125}I-LDL released from the surface when the cells were subsequently incubated with dextran sulfate. From Kovanen *et al.* (1979b).

caused an increase in LDL receptor activity in the mouse Y-1 adrenal cells, whether the cells were grown in the absence or in the presence of LDL. The observed regulation of LDL receptor activity in these cultured adrenal cells was consistent with the pattern previously observed in fibroblasts. In the presence of LDL, cells develop an amount of receptor activity that is just sufficient to supply the cholesterol needed for growth and metabolic purposes. Maximal LDL receptor levels develop only when there is an enhanced demand for cholesterol or when plasma LDL is not available (Brown and Goldstein, 1975).

2. Membranes Prepared from Fresh Bovine Adrenal Cortex

The demonstration of functional LDL receptors in cultured bovine adrenocortical cells laid the foundation for the study of such receptors in membranes prepared from fresh adrenal cortex. To demonstrate high-affinity binding in such isolated membranes, we used an ultracentrifugation assay that had been shown to measure cell surface receptor activity in membranes prepared from cultured

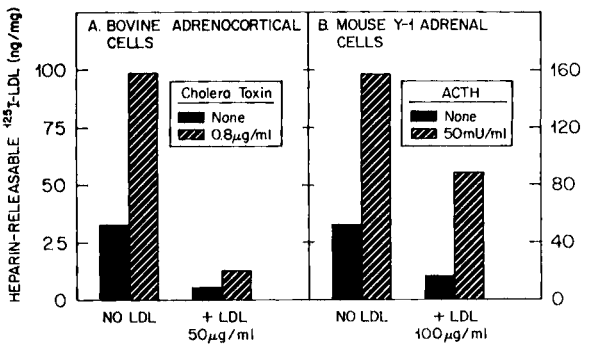

FIG. 16. Regulation of low-density lipoprotein (LDL) receptor activity in bovine adrenocortical cells (A) and in mouse Y-1 adrenal cells (B) by prior incubation with cholera toxin (A) or ACTH (B) in the presence and in the absence of LDL. The cells were incubated for either 24 hours (A) or 48 hours (B) in medium containing the indicated addition. They were then washed thoroughly and incubated for 3 hours with ^{125}I-labeled LDL at 37°C. The amount of ^{125}I-labeled LDL bound to the receptor was determined with the heparin release assay.

human fibroblasts (Basu *et al.*, 1978). In this assay, cells are homogenized with a Polytron homogenizer and the membranes sedimenting between 8000 *g* and 100,000 *g* are isolated. The membranes are incubated with ^{125}I-LDL, and the bound and free lipoproteins are separated by centrifugation at 100,000 *g* using an air-driven ultracentrifuge (Kovanen *et al.*, 1979a).

To demonstrate that this assay was applicable to adrenal membranes, we prepared membranes from monolayers of cultured bovine adrenocortical cells under conditions in which the number of receptors could be estimated from studies of LDL metabolism in the intact cells. The data in Fig. 17A show that when these membranes were incubated with increasing concentrations of ^{125}I-LDL *in vitro*, saturable and high-affinity binding of the ^{125}I-LDL to the membranes could be demonstrated. The dashed line in Fig. 17A shows this high-affinity binding, which is defined as the difference between the ^{125}I-LDL binding observed in the absence of excess unlabeled LDL (total binding) and the ^{125}I-LDL binding observed in the presence of an excess of unlabeled LDL (nonspecific binding). The amount of high-affinity binding in the isolated membrane fraction was similar to the amount detected by incubation of the intact monolayers with ^{125}I-LDL (Kovanen *et al.*, 1979a,b).

Figure 17B shows the results of the same assay performed on the membrane fraction isolated from fresh bovine adrenal cortex. The affinity of the binding site for ^{125}I-LDL was similar in the membranes from the cultured cells and in the membranes from fresh tissue. Like the LDL receptor in the cultured cells, the binding site in the membranes from fresh tissue was shown to bind bovine or human LDL, but not human or bovine HDL (Kovanen *et al.*, 1979a,b). The LDL binding site in fresh membranes also resembled the LDL receptor in the cultured

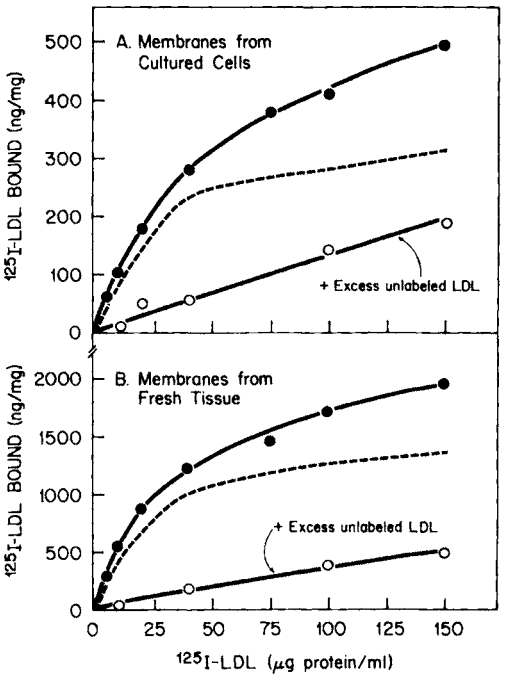

FIG. 17. Concentration dependence of ^{125}I-labeled low-density lipoprotein (^{125}I-LDL) binding to bovine adrenocortical membranes prepared from cultured cells (A) and from fresh tissue (B). Membranes were prepared from cultured bovine adrenocortical cells grown in the absence of LDL and from fresh bovine adrenal cortex. Each reaction tube contained 145 μg of membrane protein and the indicated concentration of human ^{125}I-LDL (245 cpm/ng) in the absence (●) or in the presence (○) of 1 mg/ml of unlabeled human LDL. After incubation for 40 minutes at 0°, the amount of ^{125}I-LDL bound to the membranes was determined by ultracentrifugation assay. The dashed line shows the high-affinity binding, which was calculated by subtracting the amount of ^{125}I-LDL bound in the presence of the excess unlabeled LDL from that bound in the absence of the excess unlabeled LDL. From Kovanen *et al.* (1979a).

bovine adrenocortical cells in two other respects: it was susceptible to destruction by Pronase, and the binding required a divalent cation, either calcium or manganese (Kovanen *et al.*, 1979a).

The data in Fig. 18A show that the amount of ^{125}I-LDL bound to membranes of adrenal cortex was proportional to the amount of membrane protein added to the assay. High-affinity binding of ^{125}I-LDL was undetectable in bovine erythrocytes, which were used as a control (Fig. 18B).

We concluded from these studies that membranes from the bovine adrenal cortex expressed a specific high-affinity LDL binding site that was similar to the functional LDL receptor that had been demonstrated in the cultured bovine adrenocortical cells. The data strongly suggested that *in vivo* this LDL receptor supplied cholesterol to the adrenal cortex for steroid hormone synthesis.

To test this hypothesis in another way, we compared the LDL binding activity

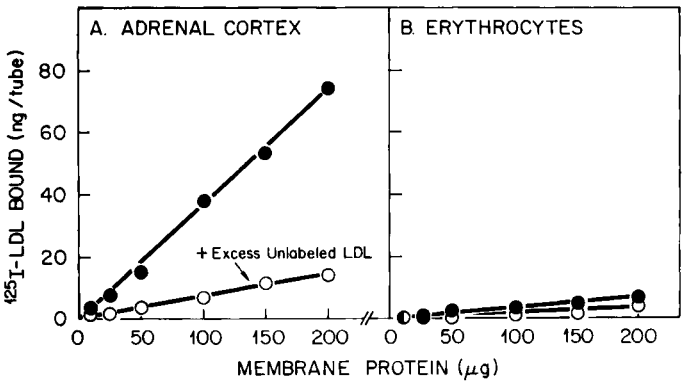

FIG. 18. Comparison of ^{125}I-labeled low-density lipoprotein (^{125}I-LDL) binding to membranes from bovine adrenal cortex (A) and from bovine erythrocytes (B). Each reaction tube contained the indicated amount of membrane protein and 12.5 μg/ml of bovine ^{125}I-LDL (988 cpm/ng in panel A and 507 cpm/ng in panel B) in the absence (●) or in the presence (○) of 500 μg of unlabeled LDL per milliliter. After incubation at 0° for 40 minutes, the amount of ^{125}I-LDL bound to the membranes was determined by ultracentrifugation assay. From Kovanen *et al.* (1979a).

in the membranes of adrenal cortex with that of membrane fractions prepared from 17 other tissues of the cow. The data in Fig. 19 show that the amount of high-affinity binding per milligram of membrane protein was 6 times higher in the adrenal cortex than in the medulla of the same glands. Similarly, the amount of high-affinity LDL binding was much greater in the corpus luteum than in the ovarian interstitium. The occurrence of large amounts of LDL binding activity in these two steroid hormone-secreting tissues lent strong support to the thesis that this activity represented an LDL receptor that was supplying cholesterol for steroid hormone synthesis.

Although the amounts of binding in the other tissues were lower than those of the adrenal cortex and corpus luteum, definite high-affinity ^{125}I-LDL binding activity could be demonstrated in membranes from adipose tissue, myocardium, skeletal muscle, thymus, kidney, lung, ileum, jejunum, and testes. In the liver, there was a large amount of nonspecific binding of ^{125}I-LDL, and this precluded an accurate assessment of the amount of high-affinity binding (Kovanen *et al.*, 1979a).

D. THE FOURTH MODEL: HUMAN ADRENAL MEMBRANES

The availability of an assay for high-affinity ^{125}I-LDL binding to tissue membranes has allowed us to begin to study the distribution of ^{125}I-LDL binding activity in human tissues. As a first step, we have studied this activity in a series of human fetuses that were obtained as a result of spontaneous abortion between weeks 16 and 20 of pregnancy. Figure 20A shows that membranes prepared from

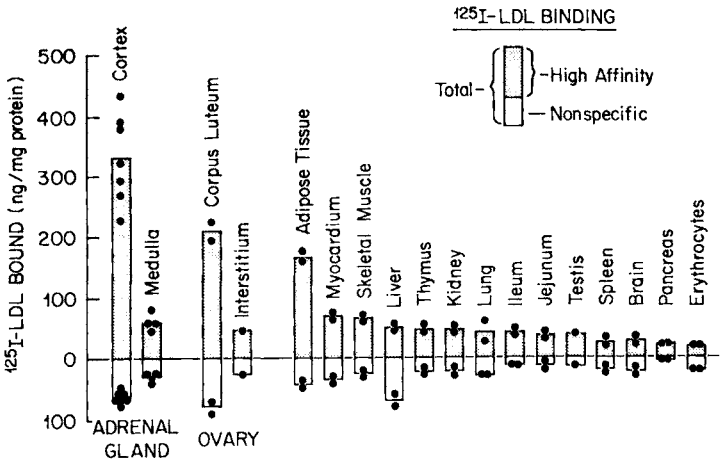

FIG. 19. Comparison of ^{125}I-labeled low-density lipoprotein (^{125}I-LDL) binding activity to membranes prepared from different bovine tissues. The indicated bovine tissues were homogenized, and the 8000 *g* to 100,000 *g* membrane pellet was isolated. Each reaction tube contained 75 μg of membrane protein and 12.5 μg of bovine ^{125}I-LDL per milliliter (500–900 cpm/ng) in the absence or in the presence of 500 μg/ml of unlabeled bovine LDL. After incubation at 0° for 40 minutes, the amount of ^{125}I-LDL bound to the membranes was determined by ultracentrifugation assay. Total binding represents the amount of ^{125}I-LDL bound to the membranes in the absence of excess unlabeled LDL. High-affinity binding and nonspecific binding are those components of the total binding that were, respectively, inhibited and not inhibited competitively by the presence of the excess unlabeled LDL. Each point represents the average of duplicate assays performed on the membranes obtained from one animal. Data from Kovanen *et al.* (1979a).

one human fetal adrenal gland bound ^{125}I-LDL with high affinity and saturability. Binding of the radioactive ligand was inhibited competitively by unlabeled LDL. High-affinity binding of ^{125}I-HDL to the same membranes was also detected, but the amount of such binding was much lower than that for LDL (Fig. 20B).

The specificity of the ^{125}I-LDL binding site in the human fetal adrenal membranes was tested by means of competition studies. The data in Fig. 21 show that unlabeled LDL competed with ^{125}I-LDL for binding to the membranes, 50% competition occurring at an LDL concentration of approximately 10 μg of protein per milliliter. In contrast, HDL did not achieve 50% competition, even at concentrations as high as 500 μg of protein per milliliter.

High-affinity ^{125}I-LDL binding could also be demonstrated in membranes prepared from the fetal testis (Fig. 22A). Distinct saturable ^{125}I-LDL binding was also detected in membranes prepared from two fetal livers (Fig. 22B). The relative amount of binding in the fetal liver membranes was 5-fold lower than that in the membranes from the fetal testis. The data in Fig. 23 show the amounts of ^{125}I-LDL binding activity per milligram of membrane protein in various tissues of the human fetus. The binding activity in the adrenal gland was 30-fold higher than that seen in any other tissue except the gonad (testis). This distribution of

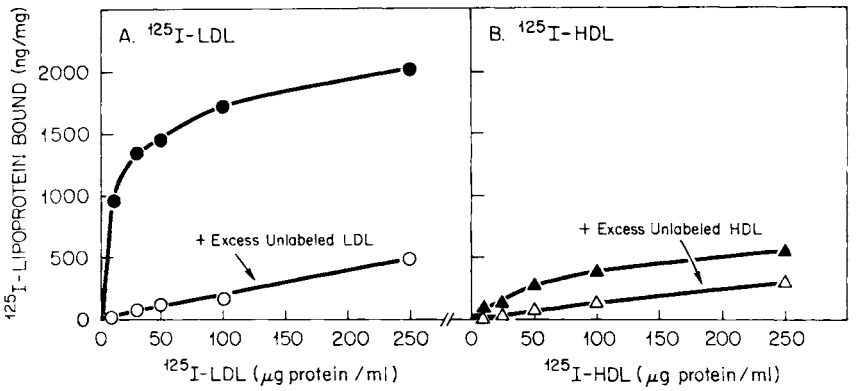

FIG. 20. Concentration dependence of ^{125}I-labeled low-density lipoprotein (^{125}I-LDL) binding (A) and of ^{125}I-labeled high-density lipoprotein (^{125}I-HDL) binding (B) to human fetal adrenal membranes. Membranes from the adrenal gland of a 16-week-old human fetus were prepared, and binding assays were performed as described by Kovanen *et al.* (1979a). Each reaction tube contained 75 μg of membrane protein and the indicated concentration of human ^{125}I-LDL (280 cpm/ng) in the absence (●) or in the presence (○) of 1 mg of unlabeled human LDL per milliliter (panel A) or ^{125}I-HDL (350 cpm/ng) in the absence of (▲) or in the presence (△) of 1 mg of unlabeled HDL per milliliter (panel B). After incubation for 40 minutes at 0°C, the amount of ^{125}I-labeled lipoprotein bound to the membranes was determined by ultracentrifugation assay.

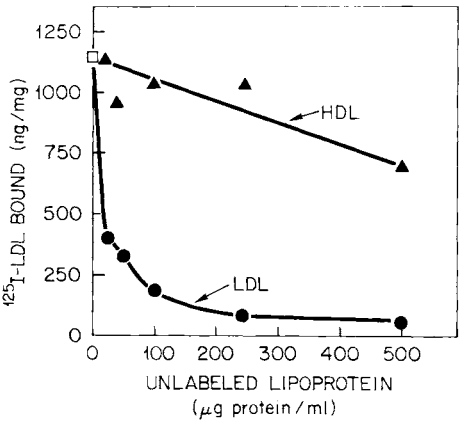

FIG. 21. Comparison of the ability of low-density lipoprotein (LDL) (●) and high-density lipoprotein (HDL) (▲) to compete with ^{125}I-labeled LDL for binding to membranes from human fetal adrenal gland. Membranes from the adrenal gland of a 16-week-old human fetus were prepared and binding assays were performed as described by Kovanen *et al.* (1979a). Each reaction tube contained 75 μg of membrane protein, 12.5 μg of ^{125}I-LDL per milliliter (237 cpm/ng), and the indicated concentration of either unlabeled LDL (●) or HDL (▲). After incubation at 0° for 40 minutes, the amount of ^{125}I-LDL bound to the membranes was determined by ultracentrifugation assay.

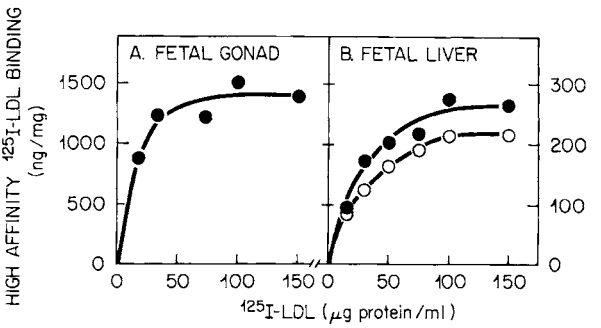

FIG. 22. Concentration dependence of ^{125}I-labeled low-density lipoprotein (^{125}I-LDL) binding to membranes from human fetal gonads (A) and liver (B). Membranes were prepared from the gonads (testes) and liver of a 16-week-old male fetus (●). Membranes were also prepared from the liver of a 20-week-old fetus (○). Binding assays were performed as described by Kovanen *et al.* (1979a). Each reaction tube contained either 17 μg (gonad) or 75 μg (liver) of membrane protein and the indicated concentration of ^{125}I-LDL (234 cpm/ng) in the absence or in the presence of 1 mg of unlabeled LDL per milliliter. After incubation for 40 minutes at 0°C, the amounts of total and nonspecific ^{125}I-LDL binding to the membranes were determined by ultracentrifugation assay. The values for high-affinity binding were calculated by subtracting the amount of ^{125}I-LDL bound in the presence of excess unlabeled LDL from that bound in the absence of the excess unlabeled LDL.

^{125}I-LDL binding activity was similar to the results previously obtained with the adult cow (Kovanen *et al.*, 1979a).

We also measured HMG-CoA reductase activity as an index of the capacity for cholesterol synthesis in the various human fetal tissues. The data in Fig. 24 show that HMG-CoA reductase activity was extremely high in the microsomes prepared from the human fetal adrenal and the gonad (testis). The finding of high HMG-CoA reductase activity in the same steroid-secreting tissues that showed high ^{125}I-LDL receptor activity suggests that these two tissues have a large requirement for cholesterol. The fetal liver also had a relatively high HMG-CoA reductase activity, whereas enzyme activity in the other human fetal tissues was low (Fig. 24).

The data in Fig. 25 compare the binding of ^{125}I-LDL to membranes prepared from the adrenal gland and liver of several different species. These assays were all performed under one set of conditions in which the tissue membranes were incubated with human ^{125}I-LDL at 12.5 μg of protein per milliliter in the absence or in the presence of an excess of unlabeled human LDL. The data show that the amount of high-affinity ^{125}I-LDL binding was highest in the adrenal of the human fetus. High levels of binding activity were also seen in the adrenal of the dog, cow, and swine. In all these species the binding was much greater in the adrenal gland than in the liver. On the other hand, no significant high-affinity binding activity was detected in the adrenal gland of the rabbit and the rat. The mouse adrenal showed a low but clearly detectable level of high-affinity ^{125}I-LDL binding activity.

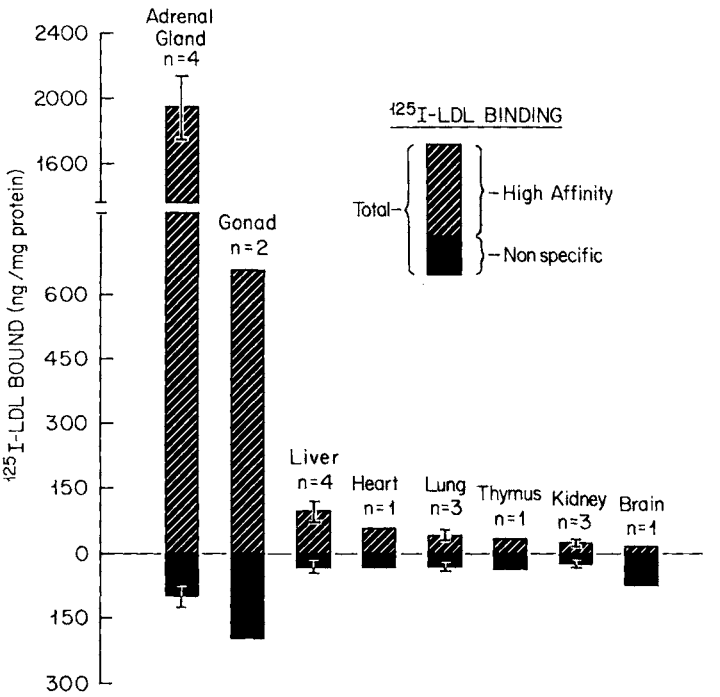

FIG. 23. Comparison of ^{125}I-labeled low-density lipoprotein (^{125}I-LDL) binding activity in human fetal membranes prepared from various organs. Membranes were prepared from 16- to 20-week-old human fetuses as described by Kovanen *et al.* (1979a) and incubated with human ^{125}I-LDL as described in the legend to Fig. 19. High-affinity and nonspecific binding were calculated as described in the legend to Fig. 19. All the gonads were testes. Each bar represents the mean ± 1 SEM of results obtained from the indicated number of human fetuses.

Considered together with the other data in this review, the data in Fig. 25 are consistent with the thesis that the adrenal gland of the human fetus, the dog, the cow, swine, and perhaps the mouse rely on LDL for a large part of their steroid hormone production. In contrast, the evidence for high-affinity binding of human ^{125}I-LDL in adrenal tissues from the rabbit and the rat is not convincing. These species differences would appear to explain the difference between cholesterol metabolism in the cultured mouse Y-1 adrenal cells and bovine adrenocortical cells on the one hand and the rat treated with 4-APP on the other. Cells from the mouse and the cow appear to express LDL receptor activity in culture that is equivalent to that occurring *in vivo*. On the other hand, we have yet to find evidence for a specific LDL receptor in membranes from the rat adrenal gland. The mechanism for cholesterol uptake from lipoproteins in this species may well differ from that of the other species studied and account for the fact that HDL as well as LDL appear to supply cholesterol to the adrenal of the 4-APP-treated rat in contrast to the preferential utilization of LDL in other species.

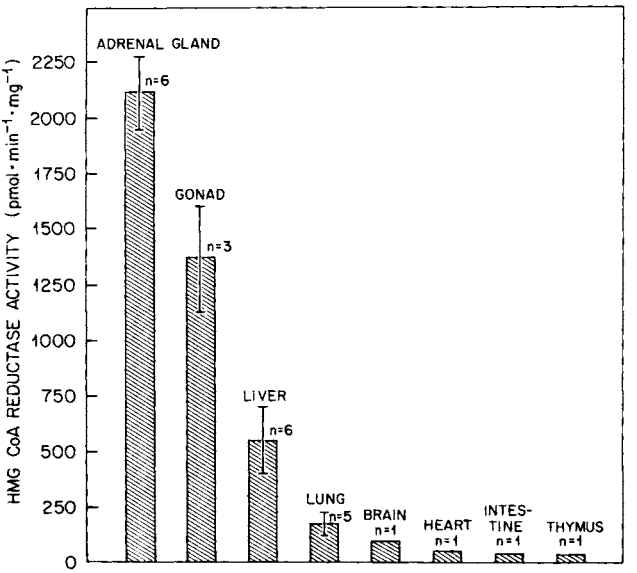

FIG. 24. Comparison of microsomal HMG-CoA reductase activity in various human fetal tissues. Fresh fetal tissues were homogenized in a Dounce homogenizer, microsomal fractions were prepared, and HMG-CoA reductase was assayed as previously described by Kovanen *et al.* (1978). Each bar represents the mean ± 1 SEM of results obtained from the indicated number of human fetuses. The ages of the fetuses ranged from 16 to 22 weeks. All fetuses were male.

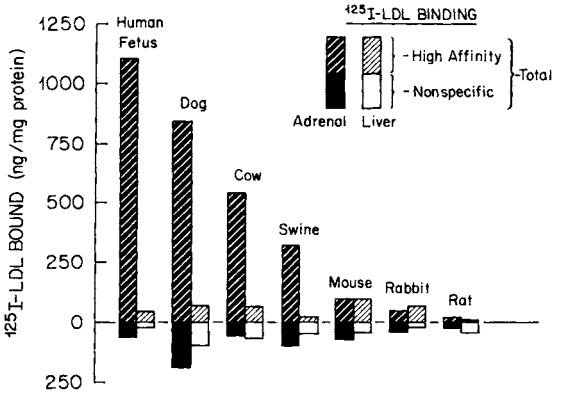

FIG. 25. Comparison of ^{125}I-labeled low-density protein (^{125}I-LDL) binding activity in adrenal and liver membranes from various species. Membranes were prepared according to the method of Kovanen *et al.* (1979a). The membranes were prepared either from whole adrenal gland (human fetus, mouse, rabbit, and rat) or from adrenal cortex (dog, cow, and swine). The membranes were incubated with 12.5 μg of human ^{125}I-LDL per milliliter in the presence or in the absence of 500 μg of unlabeled human LDL per milliliter as described in the legend to Fig. 19. High-affinity binding and nonspecific binding were calculated as described in the legend to Fig. 19.

III. Summary: A Working Model for Cholesterol Metabolism in the Adrenal Cortex

The studies in the four model systems discussed above allow the formulation of a hypothetical working model to explain some aspects of cholesterol metabolism in the adrenal cortex. In this model, which is shown schematically in Fig. 26, the adrenal is considered to have a small pool of metabolically active free cholesterol that is rapidly turning over. In the steady state, the input and output of cholesterol from this metabolically active cholesterol pool must be balanced. The net input into this pool comes from three sources: (1) uptake of cholesterol from lipoproteins; (2) endogenous synthesis of cholesterol within the gland; and (3) hydrolysis of stored cholesteryl esters. Net output of cholesterol from this pool occurs when cholesterol is converted to steroid hormones that are secreted from the gland and when cholesterol is esterified to form cholesteryl ester droplets (Fig. 26A).

The adrenal gland contains at least two pools of cholesterol in addition to the

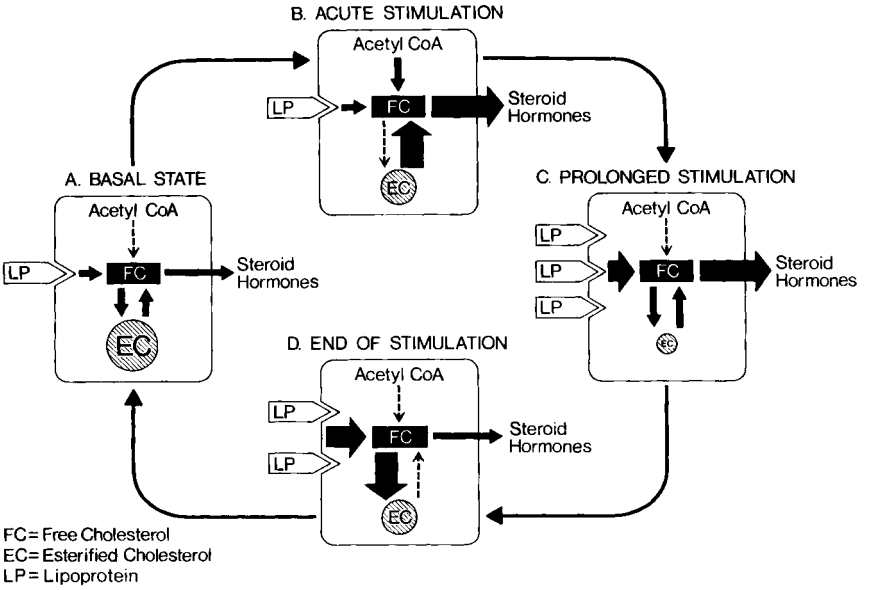

FIG. 26. Model for cholesterol homeostasis in the adrenal gland, showing sequential changes in cholesterol input and output during acute and prolonged stimulation of steroid secretion. This model is based on a compilation of the data obtained in the cultured mouse Y-1 cells, the cultured bovine adrenocortical cells, and the rat treated with 4-aminopyrazolopyrimidine (4-APP). In the two-cell culture systems that have been studied, low-density lipoprotein is the lipoprotein that specifically delivers cholesterol to the adrenal gland. The lipoprotein specificity for the cholesterol delivery process in the rat adrenal has not yet been elucidated. See text for details. LP, lipoprotein; FC, free cholesterol; EC, esterified cholesterol.

metabolically active pool. One of these is a fixed pool of free cholesterol in cell membranes. Studies in tissue culture indicate that this pool of membrane cholesterol is not available (in a net sense) to provide cholesterol for steroid synthesis. The second pool of cholesterol is contained in storage droplets, where the cholesterol is esterified with fatty acids. As discussed below, these cholesteryl esters exert a buffer function that tends to stabilize the free cholesterol content of the adrenal gland during transient fluctuations in steroid demand.

When the adrenal gland is stimulated acutely with ACTH, the initial response is a rapid output of steroids (Fig. 26B). The substrate for this initial burst of synthesis comes from cholesterol that is drained from the small pool of metabolically active free cholesterol in the gland. This sterol is immediately replenished by the hydrolysis of stored cholesteryl esters. In addition, cholesterol synthesis is activated through an enhancement in the activity of HMG-CoA reductase (and HMG-CoA synthase in the rat). If the stimulus to steroid secretion is prolonged, net cholesteryl ester hydrolysis eventually ceases, cholesterol synthesis declines, and the enhanced cholesterol output becomes balanced primarily by an accelerated uptake of cholesterol from plasma lipoproteins (Fig. 26C). Data from the two adrenal cell culture systems (mouse and bovine) as well as from the 4-APP-treated rat indicate that as long as plasma cholesterol is available the bulk of the cholesterol used to support steroid synthesis over the long term comes from plasma lipoproteins, and only a minor portion comes from endogenous cholesterol synthesis. In the cultured mouse and bovine cells, the enhanced uptake of lipoprotein-cholesterol occurs as the result of an increase in the number of cellular LDL receptors.

When the number of LDL receptors is adequate and when the plasma concentration of LDL is normal, sufficient cholesterol enters the adrenal cell during prolonged stimulation to support a high rate of steroid synthesis (Fig. 26C). The cellular content of cholesteryl esters is variable, depending on whether lipoprotein uptake is greater than or less than the steroid secretion rate. When the stimulus to steroid secretion ceases, a transient situation exists in which lipoprotein uptake exceeds steroid output. During this interval, the cholesteryl esters of the adrenal are restored (Fig. 26D), after which the LDL receptors become suppressed and the gland returns to a basal state (Fig. 26A).

In the model systems so far studied, endogenous synthesis of cholesterol in the adrenal is important in several situations: (1) when insufficient plasma lipoproteins are available, a situation that is probably never encountered in normal physiology; (2) transiently, when there is a sudden stimulus to steroid secretion and sufficient time has not elapsed for full induction of lipoprotein receptor activity; and (3) when the rate of steroid synthesis is so great that maximal lipoprotein receptor activity cannot supply sufficient cholesterol and supplementary cholesterol synthesis within the gland is required.

The strongest evidence for the role of LDL receptors in the adrenal lipoprotein uptake process comes from the studies of cultured adrenal cells. In the mouse and

bovine adrenal cells, LDL receptors have been demonstrated directly to supply the bulk of the cholesterol for steroid synthesis. Numerous types of independent evidence have documented the existence of functional LDL receptors in these two cultured cell systems. The evidence that similar LDL receptors also function in the adrenal cortex *in vivo* is indirect and rests on two observations: (1) that LDL binding sites similar to those in the cultured mouse and bovine cells can be demonstrated in isolated membranes from the adrenal cortex of these two species, and (2) that the amount of LDL binding activity in steroid-secreting organs of the cow and the human fetus is much higher than that in other organs. The results in the 4-APP-treated rat support the notion that the adrenal gland relies on plasma cholesterol, although the mechanism for lipoprotein uptake in this species has not yet been clarified. It is possible that under some circumstances *in vivo* adrenal cells of the rat and perhaps other species may express a mechanism in addition to the LDL receptor that allows them to derive cholesterol from HDL.

In addition to the need for *in vivo* demonstration of the function of LDL and possibly HDL receptors in intact adrenals, several key questions remain unresolved: (1) What is the mechanism by which the adrenal gland senses the content of metabolically active cholesterol and thereby coordinately regulates lipoprotein receptor activity, cholesterol synthesis, and cholesteryl ester formation? (2) What is the mechanism by which cholesterol is transported from one cellular compartment (i.e., the lysosome) to another compartment (i.e., the mitochondrion)? Are cytoplasmic transport proteins involved? (3) If LDL receptors are important in normal human adrenal physiology, why is adrenal insufficiency not ordinarily seen in patients who have no circulating LDL (abetalipoproteinemia) or in patients who have a genetic deficiency of LDL receptors (familial hypercholesterolemia)? Can HDL somehow supply adrenal cholesterol in these patients, or do they derive all of their adrenal steroids from cholesterol synthesized within the gland? The answers to these and other questions should provide deeper insights into the important role of lipoprotein receptors in adrenal physiology.

ACKNOWLEDGMENTS

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REFERENCES

Albers, J. J., and Bierman, E. L. (1976). *Biochim. Biophys. Acta* **424,** 422.
Andersen, J. M., and Dietschy, J. M. (1976). *Biochem. Biophys. Res. Commun.* **72,** 880.
Andersen, J. M., and Dietschy, J. M. (1977). *J. Biol. Chem.* **252,** 3652.

Anderson, R. G. W., Goldstein, J. L., and Brown, M. S. (1976). *Proc. Natl. Acad. Sci. U.S.A.* **73,** 2434.

Anderson, R. G. W., Brown, M. S., and Goldstein, J. L. (1977). *Cell* **10,** 351.

Balasubramaniam, S., Goldstein, J. L., Faust, J. R., and Brown, M. S. (1976). *Proc. Natl. Acad. Sci. U.S.A.* **73,** 2564.

Balasubramaniam, S., Goldstein, J. L., Faust, J. R., Brunschede, G. Y., and Brown, M. S. (1977b). *J. Biol. Chem.* **252,** 1771.

Balasubramaniam, S., Goldstein, J. L., and Brown, M. S. (1977a). *Proc. Natl. Acad. Sci. U.S.A.* **74,** 1421.

Basu, S. K., Goldstein, J. L., and Brown, M. S. (1978). *J. Biol. Chem.* **253,** 3852.

Bersot, T. P., Mahley, R. W., Brown, M. S., and Goldstein, J. L. (1976). *J. Biol. Chem.* **251,** 2395.

Bilheimer, D. W., Ho, Y. K., Brown, M. S., Anderson, R. G. W., and Goldstein, J. L. (1978). *J. Clin. Invest.* **61,** 678.

Bloch, K. (1945). *J. Biol. Chem.* **157,** 661.

Borkowski, A. J., Levin, S., Delcroix, C., and Klastersky, J. (1970). *J. Appl. Physiol.* **28,** 42.

Borkowski, A., Delcroix, C., and Levin, S. (1972a). *J. Clin. Invest.* **51,** 1664.

Borkowski, A., Delcroix, C., and Levin, S. (1972b). *J. Clin. Invest.* **51,** 1679.

Bradley, W. A., Rohde, M. F., Gotto, A. M., and Jackson, R. L. (1978). *Biochem. Biophys. Res. Commun.* **81,** 928.

Brown, M. S., and Goldstein, J. L. (1974). *Proc. Natl. Acad. Sci. U.S.A.* **71,** 788.

Brown, M. S., and Goldstein, J. L. (1975). *Cell* **6,** 307.

Brown, M. S., and Goldstein, J. L. (1976). *Science* **191,** 150.

Brown, M. S., and Goldstein, J. L. (1979). *Harvey Lect.* **73,** 163.

Brown, M. S., Dana, S. E., and Goldstein, J. L. (1973). *Proc. Natl. Acad. Sci. U.S.A.* **70,** 2162.

Brown, M. S., Dana, S. E., and Goldstein, J. L. (1974). *J. Biol. Chem.* **249,** 789.

Brown, M. S., Dana, S. E., and Goldstein, J. L. (1975a). *Proc. Natl. Acad. Sci. U.S.A.* **72,** 2925.

Brown, M. S., Dana, S. E., and Goldstein, J. L. (1975b). *J. Biol. Chem.* **250,** 4025.

Brown, M. S., Faust, J. R., and Goldstein, J. L. (1975c). *J. Clin. Invest.* **55,** 783.

Brown, M. S., Anderson, R. G. W., and Goldstein, J. L. (1977). *J. Supra. Struct.* **6,** 85.

Buonassisi, V., Sato, G., and Cohen, A. I. (1962). *Proc. Natl. Acad. Sci. U.S.A.* **48,** 1184.

Deutsch, D. G., Heinrikson, R. L., Foreman, J., and Scanu, A. M. (1978). *Biochim. Biophys. Acta* **529,** 342.

Dexter, R. N., Fishman, L. M., and Ney, R. L. (1970). *Endocrinolology* **87,** 836.

Dietschy, J. M. (1978). Personal communication.

Dietschy, J. M., and Wilson, J. D. (1970). *N. Engl. J. Med.* **282,** 1128, 1179, 1241.

Faust, J. R., Goldstein, J. L., and Brown, M. S. (1977). *J. Biol. Chem.* **252,** 4861.

Fredrickson, D. S., Goldstein, J. L., and Brown, M. S. (1978). *In* "The Metabolic Basis of Inherited Disease" (J. B. Stanbury, J. B. Wyngaarden, and D. S. Fredrickson, eds.), p. 604. McGraw-Hill, New York.

Goldstein, J. L., and Brown, M. S. (1974). *J. Biol. Chem.* **249,** 5153.

Goldstein, J. L., and Brown, M. S. (1976). *Curr. Topics Cell. Reg.* **11,** 147.

Goldstein, J. L., and Brown, M. S. (1977). *Annu. Rev. Biochem.* **46,** 897.

Goldstein, J. L., and Brown, M. S. (1978). *Johns Hopkins Med. J.* **143,** 8.

Goldstein, J. L., Dana, S. E., and Brown, M. S. (1974). *Proc. Natl. Acad. Sci. U.S.A.* **71,** 4288.

Goldstein, J. L., Brunschede, G. Y., and Brown, M. S. (1975a). *J. Biol. Chem.* **250,** 7854.

Goldstein, J. L., Dana, S. E., Faust, J. R., Beaudet, A. L., and Brown, M. S. (1975b). *J. Biol. Chem.* **250,** 8487.

Goldstein, J. L., Basu, S. K., Brunschede, G. Y., and Brown, M. S. (1976). *Cell* **7,** 85.

Gospodarowicz, D., Ill, C. R., Hornsby, P. J., and Gill, G. N. (1977). *Endocrinology* **100,** 1080.

Gwynne, J. T., Mahaffee, D., Brewer, H. B., and Ney, R. L. (1976). *Proc. Natl. Acad. Sci. U.S.A.* **73,** 4329.

Havel, R. J., Goldstein, J. L., and Brown, M. S. (1979). *In* "Diseases of Metabolism" (P. K. Bondy and L. E. Rosenberg, eds.). Saunders, Philadelphia, Pennsylvania. In press.
Hechter, O., Zaffaroni, A., Jacobson, R. P., Levy, H., Jeanloz, R. W., Schenker, V., and Pincus, G. (1951). *Recent Prog. Horm. Res.* **6,** 215.
Henderson, J. F. (1963). *J. Lipid Res.* **4,** 68.
Ho, Y. K., Brown, M. S., Bilheimer, D. W., and Goldstein, J. L. (1976a). *J. Clin. Invest.* **58,** 1465.
Ho, Y. K., Brown, M. S., Kayden, H. J., and Goldstein, J. L. (1976b). *J. Exp. Med.* **144,** 444.
Ho, Y. K., Faust, J. R., Bilheimer, D. W., Brown, M. S., and Goldstein, J. L. (1977). *J. Exp. Med.* **145,** 1531.
Hornsby, P. J., and Gill, G. N. (1977). *J. Clin. Invest.* **60,** 342.
Hornsby, P. J., and Gill, G. N. (1978). *Endocrinology* **102,** 926.
Jackson, R. L., Morrisett, J. D., and Gotto, A. M. (1976). *Physiol. Rev.* **56,** 259.
Kane, J. P. (1977). *In* "Lipid Metabolism in Mammals" (F. Synder, ed.), Vol. I, p. 209. Plenum, New York.
Kovanen, P. T., Goldstein, J. L., and Brown, M. S. (1978). *J. Biol. Chem.* **253,** 5126.
Kovanen, P. T., Basu, S. K., Goldstein, J. L., and Brown, M. S. (1979a). *Endocrinology* **104,** 610.
Kovanen, P. T., Faust, J. R., Brown, M. S., and Goldstein, J. L. (1979b). *Endocrinology* **104,** 599.
Kowal, J. (1970). *Recent Prog. Horm. Res.* **26,** 623.
Krieger, M., Brown, M. S., Faust, J. R., and Goldstein, J. L. (1978). *J. Biol. Chem.* **253,** 4093.
Kuehl, K. S., Ramm, L. E., and Langdon, R. G. (1977). *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **36,** 828 (abstract).
Mahley, R. W. (1978). *In* "Disturbances in Lipid and Lipoprotein Metabolism" (J. M. Dietschy, A. M. Gotto, and J. A. Ontko, eds.), p. 181. Am. Physiol. Soc., Bethesda, Maryland.
Mahley, R. W., Innerarity, T. L., Pitas, R. E., Weisgraber, K. H., Brown, J. H., and Gross, E. (1977). *J. Biol. Chem.* **252,** 7279.
Morris, M. D., and Chaikoff, I. L. (1959). *J. Biol. Chem.* **234,** 1095.
Shireman, R., Kilgore, L. L., and Fisher, W. R. (1977). *Proc. Natl. Acad. Sci. U.S.A.* **74,** 5150.
Simonian, M., Hornsby, P. J., and Gill, G. N. (1978). Personal communication.
Steinberg, D., Nestel, P. J., Weinstein, D. B., Remaut-Desmeth, M., and Chang, C. M. (1978). *Biochim. Biophys. Acta* **528,** 199.
Yasumura, H., Buonassisi, V., and Sato, G. (1966). *Cancer Res.* **26,** 529.

DISCUSSION

J. H. Clark: The first thing I think about in cholesterol synthesis and ACTH action is the depletion of cholesterol from adrenal pools. Yet you are telling me that ACTH is causing cholesterol to be taken up.

M. S. Brown: I think that one must distinguish between an acute response and a steady-state response. There is no question that acutely ACTH activates a cholesterol ester hydrolase in the adrenal gland and there is a rapid hydrolysis of cholesterol esters. Some of this free cholesterol may leak out of the gland, and some is used for steroid hormone synthesis. The initial result is a depletion of cholesterol esters. However, if you continue to follow the animal, the cholesterol esters climb back up again. If you take an intact rat and give it ACTH, and measure the cholesterol ester content of the adrenal, the cholesterol content falls and reaches a low point after about 3 hours. If you continue ACTH administration, even though steroid hormone secretion continues at maximal rates, the cholesterol esters do not fall further, and they even increase somewhat. As long as plasma cholesterol is available, we have not been able to produce a complete depletion of cholesterol esters by ACTH in the rat adrenal.

M. Sherman: Are the concentrations of heparin required to release LDL from adrenal receptors comparable to those in a patient with coronary artery disease treated with heparin? In other words, could increased cholesterol biosynthesis be an undesirable side effect of heparin treatment?

M. S. Brown: The amount of heparin required to release LDL is really enormous, and it would be much higher than you would see *in vivo* with heparin administration.

G. L. Flickinger: Dr. Brown, I would like to congratulate you for this excellent presentation. Extension of your concepts about lipoprotein receptors and their role in cholesterol metabolism to steroid-secreting cells provides us with a new framework for studying yet another mechanism whereby steroidogenesis may be regulated. Recently we have begun to examine the role of circulating cholesterol as a precursor for steroid synthesis in ovarian tissue. Our initial studies were carried out in superovulated immature rats treated with 4-APP. The findings with this model were reported at the 60th Annual Meeting of the Endocrine Society, Miami, 1978 (G. Flickinger, M. Christie, and J. Strauss, Abstract #374) and they were similar to the changes that you have described today in the adrenal of APP-treated rats.

Further studies were then undertaken to determine relationships between ovarian function and sterol metabolism throughout the lifespan of luteal tissue in superovulated rats (Fig. A). Secretion of progesterone, as assessed by plasma concentrations of this hormone, rises continuously to reach maximum levels on days 6–8 (day of hCG treatment = day 0), and thereafter it declines (Fig. A,

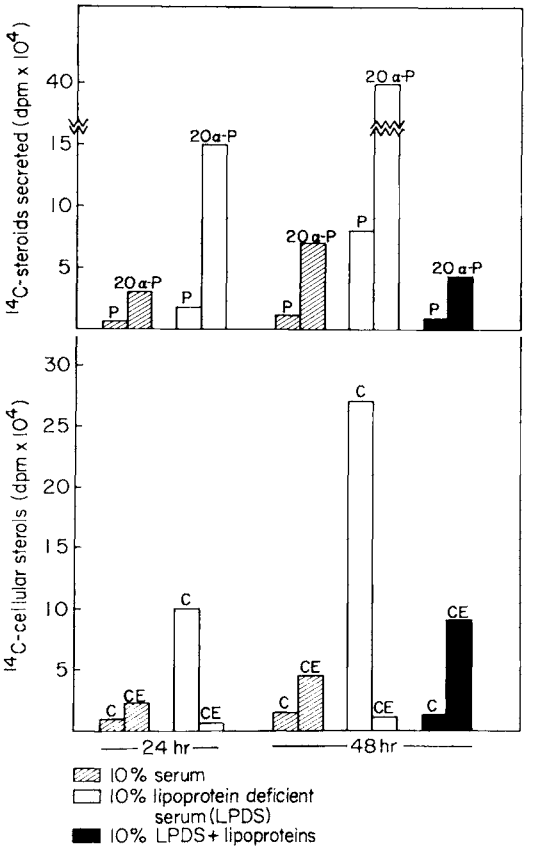

FIG. A. Relationship between ovarian function and steroid metabolism in luteal tissue of superovulated rats. P,=progesterone; 20α-P,=20α-hydroxypreg-4-3n-3-one; C,=cholesterol; CE, cholesteryl esters.

lower panel). Although the magnitude of the changes is less, the pattern of free cholesterol content of the ovary is similar to that of plasma progesterone. The low ovarian concentrations of cholesteryl ester (days 1–4) suddenly rise 6-fold between days 4 and 6. A few days later the sterol ester levels decline in conjunction with the fall in plasma progesterone. These cyclic changes were then related to the ovarian activities of HMG-CoA reductase and acyl-CoA: cholesterol acyltransferase (ACAT) (Fig. A, top panel). HMG-CoA reductase activity increased 5-fold between days 1 and 2, and thereafter it rapidly declined so that the lowest activity coincided with the period of greatest progesterone secretion and sterol accumulation. In contrast the ovarian levels of ACAT activity paralleled the changes in ovarian sterol ester concentrations. These findings, as well as those from the APP-treated rats, suggested to us that blood lipoproteins, rather than endogenous biosynthesis, served as the major source of cholesterol for progestin formation in luteal tissue of the rat. Furthermore, it would appear that blood cholesterol upon entering the luteal cells suppressed HMG-CoA reductase activity and stimulated ACAT activity as you have described today in the mouse and rat adrenal.

To examine more specifically the role of lipoproteins, we have turned to studies of rat granulosa cells in tissue culture. These cells from large preovulatory follicles of PMS-LH stimulated immature rats are cultured for 2 days in media containing 10% human male serum and ovine prolactin. During this period the cells become luteinized and they begin to secrete progestins. On day 3 the medium is changed so that it contains either 10% human serum (controls) or 10% lipoprotein-deficient serum (LPDS). At the same time ^{14}C-acetate is added to the dishes and the cells are incubated for either 24 or 48 hours prior to the measurement of radiolabeled sterols and steroids (Fig. B). In addition, certain cultures incubated in the presence of LPDS for 24 hours are then fed human serum lipoproteins (600 μg of cholesterol), and the incubations are continued for another 24 hours.

As shown in Fig. B, during the first 24 hours in culture the incorporation of [^{14}C]acetate into cellular free cholesterol and secreted progestins is enhanced when the cells are exposed to LPDS. The formation of [^{14}C]acetate for sterol and steroid synthesis between luteal cells exposed to and deprived of lipoproteins are more accentuated after 48 hours of culture. However, if cells grown in the presence of LPDS are fed lipoproteins, the incorporation of [^{14}C]acetate into free cholesterol and progestins is suppressed, while the formation of [^{14}C]cholesteryl ester is increased during the subsequent 24 hours of incubation. These findings with cultured granulosa cells are similar to your studies with the mouse Y-1 adrenal cell, and hence they support your concepts about the role of lipoproteins in the regulations of intracellular cholesterol and steroid metabolism. Whether these effects of lipoproteins on ovarian cells are mediated via receptors for lipoproteins remains to be determined.

Although you have not here presented any studies about the ovary, a recent publication from your laboratory [P. T. Kovanen, J. L. Goldstein, M. S. Brown, *J. Biol. Chem.* **253,** 5126 (1978)] described high levels of HMG-CoA reductase activity in the corpus luteum of rabbit throughout pregnancy. I would like to hear your comments on why the rabbit CL seems to differ from our findings with the luteal cell of the rat?

M. S. Brown: The data that you have shown appear quite clean and convincing. With regard to the last question: there are going to be species differences in many of these processes. I think that our finding in the rabbit corpus luteum that there was massive HMG-CoA reductase activity that was maintained throughout pregnancy may be a peculiarity of the rabbit. It would be interesting to follow this phenomenon in the pregnant rat. I imagine that what you saw with your HCG administration will take place. That is, the reductase will go up initially and eventually fall. This is really what we expected to see in the rabbit, but you know the rabbit is peculiar in that the plasma cholesterol drops to very low levels during pregnancy and it may be that for that reason the rabbit retains the ability to synthesize more cholesterol within the ovary.

The thing that would interest me with regard to your work in the rat would be whether you have compared various lipoproteins in terms of restoring the cholesterol ester content or suppressing the reductase.

G. L. Flickinger: The results that I showed with the cultures of rat granulosa cells were obtained when a lipoprotein preparation containing both LDL and HDL was added to the media. In more

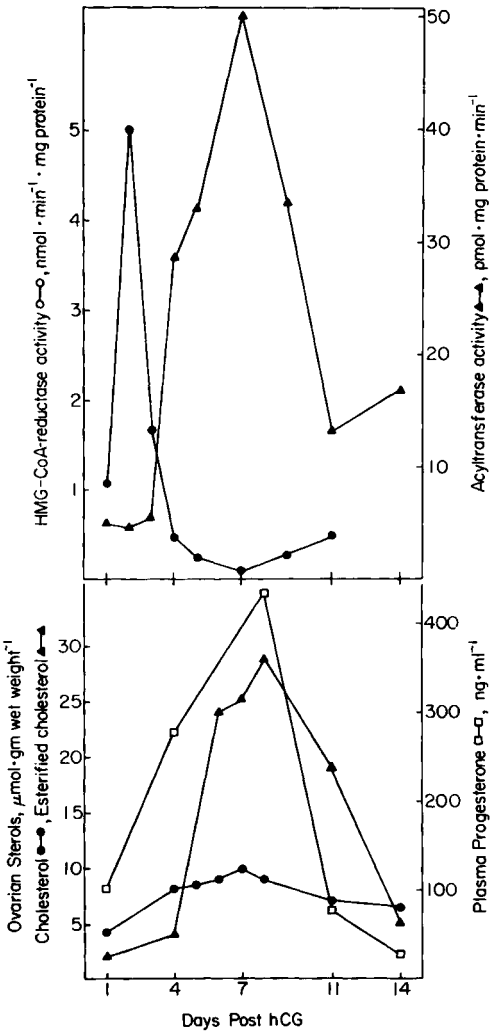

FIG. B.

recent studies we have carried out the same experiments with purified HDL and LDL. Both lipoproteins were effective in altering the utilization of [^{14}C]acetate.

M. S. Brown: That is the observation that we have made *in vivo* in the rat adrenal as well. It would be interesting to work out the specificity of that lipoprotein uptake process, and you have a system that should allow you to study the specificity of that receptor quite well.

M. D. Siperstein: I was particularly intrigued by the observation of the coordinate stimulation of HMG-CoA synthase, which probably occurs in other tissues as well. Specifically, I wanted to ask whether, if you treat with 4-APP for longer periods of time, you see the stimulation of the postmevalonate steps that is also seen in the other tissues but tends to be delayed. And second, the

ACTH stimulation of binding, or receptor, sites is rather striking and, I think, very important. If one does the same study using fibroblasts in culture, does one see any ACTH stimulation? That is obviously another way of showing the specificity of your binding or perhaps receptor sites.

M. S. Brown: To take the last one first, ACTH in fibroblasts has no effect either on the receptors or on any other aspect of cholesterol metabolism yet tested. The interesting thing is that dibutyryl cAMP does not have any effect on any of these parameters in fibroblasts where dibutyryl cAMP reproduces the ACTH effects in the adrenal cells. So it is not only the receptor, but the actual response to cAMP, that differs in fibroblasts and adrenal cells.

M. D. Siperstein: This finding I think speaks even more strongly for the specificity of the ACTH effect than does the red cell experiment.

M. S. Brown: Yes, that is true. Now with regard to the postmevalonate steps, we did not study them in these experiments. I believe that John Dietschy has not seen any change in mevalonic acid incorporation into cholesterol at a time when the reductase activity and acetate incorporation were changing rapidly. The problem with 4-APP is that one cannot extend the experiments very long because the animals become very sick, and so I do not know whether we would see changes at later time points. The capacity for incorporating mevalonate into cholesterol is relatively high. The results are reminiscent of your studies in the liver. We are struck with the fact that tissues may retain the capacity to incorporate mevalonate into cholesterol, and we do not know whether they ever use this capacity. These enzymes must be synthesized all the time, and yet it looks as though most of the time the adrenal is being supplied with cholesterol from the plasma. Therefore, HMG-CoA reductase is suppressed and these enzymes are never really utilized. Yet they are retained, perhaps for some massive response should it ever be needed. In a pinch it may be easier to induce one or two enzymes than to induce the whole pathway.

M. D. Siperstein: It just shows how important the adrenal function is.

A. White: Sayers, Fry, and Long showed a number of years ago that rat adrenal responded to ACTH *in vivo* with a dramatic, acute lowering of total cholesterol content. In view of your data showing a low response of the rat adrenal, have you tested rat adrenal cells *in vitro* to which you added low-density lipoprotein plus ACTH? Can these cells in the right environment show the higher responses you have obtained with other rat cells as well as adrenal cells from other species?

M. S. Brown: There seems to be a time lag for induction of the lipoprotein uptake mechanism in the rat adrenal. If one stimulates a rat with ACTH and follows the cholesterol ester level in the adrenal gland at hourly intervals, the cholesterol ester level is found to drop by about two-thirds and to reach a nadir at 3 hours after the start of ACTH administration. But then we find that if one continues the ACTH administration, the cholesterol esters become replenished. We believe that the initial hydrolysis of cholesterol ester seems to be necessary to cover a time period until one can induce a high rate of uptake of plasma cholesterol. Once a high rate of lipoprotein uptake is established, the lipoproteins now supply most of the cholesterol that the adrenal needs and cholesteryl esters are replenished.

A. White: In the data that you showed in your diurnal variation studies, the increase in corticoid production and alterations in the cholesterol content of the adrenal were not accompanied by alterations in the plasma levels of cholesterol. Since we generally believe that as the cholesterol level of the blood declines the liver adds more cholesterol, what is the nature of that signal in the liver? Second, since HMG-CoA reductase has been highly purified, do we know anything about the interaction of cholesterol and the enzyme?

M. S. Brown: In the rat, plasma cholesterol level is constant throughout the day. It does not show a diurnal rhythm. However, cholesterol synthesis in liver shows a peak at the midpoint of the dark cycle. Now remember that cholesterol synthesis in the liver is supplying cholesterol not only for the plasma, but also for bile acids. The relation between the peak of cholesterol synthesis and the peak of bile acid synthesis has not been well worked out. The observation is simply that one has a diurnal rhythm of hepatic cholesterol synthesis. Where the extra cholesterol goes is not clear. In regard to your second question; HMG-CoA reductase has recently been purified, and there are many exciting observations regarding this enzyme. Circumstantial evidence suggests that the enzyme may be

regulated by a phosphorylation and dephosphorylation cycle in addition to regulation of enzyme synthesis. In other words, there may be a short-term control involving a phosphorylation with resultant inactivation and a long-term control involving suppression of synthesis of the enzyme in liver. Cytosolic factors have been identified which in the presence of ATP and ADP will inactivate the HMG-CoA reductase by what appears to be a kinase reaction. The role of this inactivation in metabolism has not been well worked out yet at all.

F. Naftolin: The control of this particular intake mechanism is very interesting. Regarding the kinetics, in your Y-1 cultures it appeared that more entered, but that the rate of entry could not be seen to be very different under the influence of ACTH. How does ACTH do this? Can you in some way change the ACTH response, i.e., by regulation or autoregulation of cell membrane receptors? Also, is ACTH the only thing that does it? Have you looked at endorphins and gonadotropins in this regard?

M. S. Brown: To take first things first, I believe you are referring to the experiment in which we incubated the Y-I cells with tritiated lipoprotein (Fig. 7). If you sum the individual points at every time point, you see that the total uptake rate is about 2-fold higher in the presence of ACTH. You have to add all three forms—the esters, the free cholesterol, and the secreted steroid.

F. Naftolin: But the rate appeared to be the same?

M. S. Brown: If you look more carefully at the data you will see that the rate is 2-fold higher with ACTH. Remember, also, that we are measuring only one of several steroids produced by these cells. If we plotted the entire steroid output in the presence of ACTH, the difference would be even greater. We have not examined the question of whether other hormones regulate the receptor. It seems to us that the regulation of the receptor and the regulation of the reductase are both secondary consequences of changes in some critical cholesterol pool and are not due to a direct effect of the ACTH. For example, if we stimulate with ACTH in the presence of aminoglutethimide and block the conversion of cholesterol to steroids, we have no effect on receptors or reductase. In other words, what is required is an initial drainage of cholesterol into the mitochondria and then that is the signal for increasing cholesterol synthesis and increasing LDL receptor activity.

F. Naftolin: What happens if you pulse the ACTH three times? Do you have the same rate of LDL entry the third time as the first time?

M. S. Brown: We have not done that study.

O. Dominguez: I think that the situation with esterified cholesterol, free cholesterol, and steroid hormone production has certain important relations and several points had not been clearly seen. Actually, esterified cholesterol that drops very dramatically by ACTH is one point; the free cholesterol that is released in these circumstances perhaps does not necessarily go into steroid biosynthesis all the way through; part of it goes into the structures of the cell. A portion of esterified cholesterol that goes into a free form and remains free in the medium, without being incorporated into the membrane structures, may be exposed to a very high level of 3β-hydroxydehydrogenase isomerase and may form cholesterone, which will lose its side chain and may end excreted as coprostanol, following a metabolic pathway not turned into steroid biosynthesis. The steroid biosynthesis perhaps involves various mechanisms. Besides *de novo* synthesis from acetyl-CoA or some of the free cholesterol freshly released from esterified cholesterol, I still consider the possibility of cholesteryl sulfate as a potential precursor of steroid hormones. How many of these various alternative possibilities end in steroid hormones is something that still remains unknown.

I was wondering whether, among the esters that you are considering, other types of esters besides fatty acids, such as sulfates, are included. Cholesterol and Δ^5-3β-hydroxysteroid sulfates are also formed in the liver. It has been shown that in placenta, for example, pregnenolone sulfate from blood is the major source of progesterone biosynthesis. A similar situation could occur in the adrenal, the testis, or the ovary. Δ^5-3β-ol-steroid sulfates still may play an important role, determining and limiting, via Δ^5-3β-ol steroid sulfatase, the amount of free pregnenolone involved in hormone steroid biosynthesis. Steroid sulfatase, for example, is stimulated by ACTH through a mechanism not involving cAMP. Apparently, ATP and ADP seem to be cofactors required by the desulfation, and

they enhance the sulfatase activity. Is the cholesterol sulfate included among the esters that you are discussing, or is it not included in the picture? Otherwise, one has to find other explanations for the possible uptake of precursor sulfates that could be involved in hormone steroid biosynthesis.

M. S. Brown: We do not add any cholesterol sulfate to the culture medium. In other words, the adrenal cells in culture seem to be able to secrete steroid hormones without cholesterol sulfate. There is no cholesterol sulfate in the LDL. We have not studied the addition of cholesterol sulfate as a separate entity.

J. Kowal: In the studies performed by us to which you referred, using less sophisticated approaches, we came up with the idea that cholesterol could be synthesized in two ways in adrenal cultures. Because of our ignorance about the LDL pathway, we emphasized the importance of the endogenous synthesis route. I would like to ask a couple of questions related to this. We had also found that inhibition of steroid synthesis presented the increase in cholesterol synthesis. As a result we recognized that cholesterol was really regulating its own synthesis rather than any direct effect of ACTH. I might say that we were initially looking for some regulatable enzymes before cholesterol to pregnenolone conversion. If one holds the LDL levels constant so that it is not rate limiting, can you still see an increase in endogenous synthesis with ACTH?

M. S. Brown: Yes. If one has serum in the medium and adds ACTH, one gets stimulation of HMG-CoA reductase.

J. Kowal: Therefore, one does not have to have a deficiency of exogenous cholesterol in order to see an increase in endogenous synthesis?

M. S. Brown: That is correct. Our data measuring the reductase are similar to yours in which cholesterol synthesis was measured. When you add ACTH to the Y-1 cells you get an increase in cholesterol synthesis, even if you have maximum amounts of lipoproteins present. They use both endogenously synthesized and lipoprotein-derived cholesterol. If one quantitates the data one finds that at least 75% of the sterol is coming from the lipoproteins. The remainder is coming from synthesis.

J. Kowal: There are old data suggesting that if you expose cells, e.g., fibroblasts or adrenals, with serum containing high levels of lipoprotein the cells absorb the proteins until they explode. When we had done this we found no increase in steroid production. But a question arises from this. Is the available pool of cholesterol an absolute requirement that exogenous cholesterol enters through the receptor system, or can you blast cells with cholesterol and have that cholesterol utilized?

M. S. Brown: One can get effects by adding cholesterol in solvents such as ethanol, in which case the free cholesterol crosses the plasma membrane passively and without a requirement for the receptor. Our observation is that both in fibroblasts and in the Y-1 cells it takes massive amounts of cholesterol to reproduce the effects of small amounts of lipoproteins. You have to give enough cholesterol to practically stuff the cells with cholesterol before you turn off synthesis, whereas lipoproteins achieve this suppression at much lower levels. Clearly, the route of cholesterol delivery is important, and the lipoproteins appear to use the most efficient physiological route.

J. Kowal: And of course all this points to one thing—that the site of action of ACTH still has something to do with the conversion of cholesterol to pregnenolone. Do you have any information suggesting how the cholesterol gets into the mitochondria?

M. S. Brown: That is one of the questions that we posed at the end of the talk. We now have a new organelle with which to contend. Previously, we had the problem of hydrolyzing cholesterol esters and getting that cholesterol into the mitochondria. We now have to get the cholesterol out of the lysosome and then into the mitochondria, and how that is done is a critical question.

J. Weisz: These studies have obviously numerous important implications. They certainly identify, I think, the lysosome as an important member of the intracellular organelles that are regulating steroidogenesis by controlling the accessibility of a precursor to the steroidogenic enzymes. I was forced into looking at the lysosome in this role by one of my postdoctoral fellows, Dr. Larry Zoller. During his Ph.D. studies, Dr. Zoller noticed that when he stimulated dispersed adrenal cells with ACTH, looked at corticosone production on the one hand and changes in subcellular organelles by

morphormetric measurements at the electron microscopic level on the other, the lysosome was among the very first organelles that showed any changes. These changes occurred by 30 minutes after introduction of the ACTH [S. Malamad, L. C. Zoller, and G. J. Macdonald, *Endocrine Soc. Meet. 1976* (Abstract #388)].

During the last 2 years, working in my laboratory, Dr. Zoller has looked at this question using quantitative cytochemical techniques for measuring lysosomal membrane permeability developed by Dr. Chayen and Dr. Bitensky as used in their bioassay of TSH and presented here 3 years ago. We applied this approach to examine the changes in lysosome membrane permeability in different portions of the membrana granulosa of the preovulatory type of follicles during the last 4 days of their development, i.e., during the 4 days of the estrous cycle. Now, in the membrana granulosa of the preovulatory follicles it is the granulosa cells situated peripherally that appear to be steroidogenic. They contain lipids, Δ^5-3β-ol steroid dehydrogenase, and, as we have shown recently, cytochrome P-450. We have found a very gratifying correlation between the changes in lysosome membrane permeability in granulosa cells in the periphery during the estrous cycle and known changes in ovarian steroid secretion attributable to the follicles. Thus, lysosome membrane permeability increased during diestrus II and reached maximum in proestrus at a time when these cells may be implicated first in estrogen production and then in the progesterone surge that occurs in response to LH on the afternoon of proestrus.

I would also like to make one suggestion. One organ that might be interesting to look at in terms of the regulation of the availability of lipid precursors for steroidogenesis is the testis of the rat. Unlike other steroidogenic organs, it does not contain any lipid droplets. Of course, it does manage to put out quite a lot of testosterone, albeit in fluctuating amounts, but accumulates lipids only in the hypophectomized animals. Lipids are also present in the rat fetal testes.

B. F. Rice: A number of years ago we carried out a study with human fetal testes in tissue culture [*Steroids* **7**, 79 (1966); *Clin. Res.* **18**[2], 463 (1970)]. The tissues were grown with [1-^{14}C]acetate added to the medium. The major purpose of the study was to evaluate steroid hormone biosynthesis. A by-product of the study was an evaluation of cholesterol biosynthesis (^{14}C labeled from [1-^{14}C]-acetate, dpm) and mass of cholesterol (μg) and phytosterols (μg) in tissues and medium. We were very surprised to find that radioactive cholesterol (dpm) and nonradioactive cholesterol (μg) were being secreted into the medium. In reviewing the literature, we found that Kritchevsky at the Wistar Institute had reported biosynthesis and secretion of radioactive cholesterol by fibroblasts in tissue culture. I wonder if your final scheme should include output of cholesterol from cells as well as input of cholesterol, or do you think these data I have mentioned are artifacts of tissue culture?

M. S. Brown: It is very difficult to distinguish between net secretion and exchange of free cholesterol. If you have lipoproteins present in the culture medium, and if you incubate a cell with [^{14}C]acetate, it gets incorporated into cholesterol, and then there is a 1-to-1 exchange of labeled cholesterol molecules in the cell with cholesterol present in lipoproteins. It is really hard to sort out exchange from net secretion of cholesterol in the tissue culture system. I think in the adrenal there is evidence that net secretion of cholesterol does occur *in vivo* under some circumstances, and perhaps we should have that in our model.

H. L. Bradlow: In one of your figures on the rat studies you showed that there was a cyclic change in HMG-reductase and synthesis that peaks after the corticosterone peak and is apparently quantitatively small, like a vestigial catch-up process for cholesterol. Can you suppress this if you load up the rat with cholesterol by giving LDL as an infusion or some other process? Also, on the contrary, if you give aminoglutethimide and block the demand for cholesterol, will these cyclic responses go on? Are they autonomous responses that happen willy-nilly, or are they responses to the need for cholesterol in the gland?

M. S. Brown: Those are two excellent questions, and unfortunately we have not done either of those experiments.

A. Crastes De Paulet: Could it be possible that, in a way independent of the delivery of cholesterol to the cell, the LDL-modified adenyl cyclase activity of adrenal cells plasma membrane,

as suggested by an analog with the effect of the LDL on adipocyte plasma membrane recently shown by Chapman [*Nature* **269,** 697 (1977)], this hypothesis being enhanced by the high level of LDL binding sites in adipocyte. So my question is: Could there be some modification of the activity of adenyl cyclase in adrenal cells by LDL?

M. S. Brown: We have not studied directly any effect of LDL on adenyl cyclase. In fibroblasts we cannot find any relation between agents that affect cAMP metabolism and LDL receptor activity.

A. H. Payne: Do you believe that the difference in receptor activity in bovine testes compared to fetal testes is a species difference or difference between adult versus fetal tissue?

M. S. Brown: We really have no idea yet. I would suspect it is a fetal versus adult difference. The fetal testis, as you know, is a fairly large organ and it puts out a lot of testosterone. As I understand it, in the fetal testes, there is a much higher percentage of Leydig cells as compared with the adult. Unless you have isolated Leydig cells, it is hard to compare the measurements of activity.

A. H. Payne: Have you differentiated between receptor activity in fetal ovaries and fetal testes?

M. S. Brown: Those gonads all happened to be testes. It turns out that all the fetuses we studied were male—we have not yet studied the ovary.

K. Sterling: I believe you stated that an ethanol solution of free cholesterol would permit cholesterol to get into adrenal cells without the usual pathway of LDL receptors; my question, then, is: If we drink a little bit too much and get too high a serum ethanol concentration, is there a risk that this awful stuff (cholesterol) will be getting into our fibroblasts, our coronaries, and our myocardium by other than the usual pathway?

M. S. Brown: There is no evidence that ethanol enhances cholesterol deposition in tissues. Whatever epidemiologic evidence exists says that moderate ethanol intake somehow protects against atherosclerosis.