Serum activity and hepatic secretion of lecithin:cholesterol acyltransferase in experimental hypothyroidism and hypercholesterolemia

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Abstract Lecithin:cholesterol acyltransferase (LCAT), the major cholesterol esterifying enzyme in plasma, plays an important role in the removal of cholesterol from peripheral tissues. This study in rat focuses upon the effects of hypothyroidism and cholesterol feeding on serum activity and hepatic LCAT secretion. To obviate the effect that inclusion of high concentrations of cholesterol in the rat serum may have on the proteoliposome used in the assay of LCAT, very low and low density lipoproteins (VLDL and LDL) were removed by ultracentrifugation at d 1.063 g/ml. The molar esterification rate in the euthyroid VLDL + LDL-free serum was found to be 0.94 ± 0.06 compared to 0.67 ± 0.05 in hypothyroid rats and 1.56 ± 0.14 in hypercholesterolemic rats. LCAT secretion by suspension cultures of hepatocytes from hypercholesterolemic rats was found to be significantly depressed when compared to that for euthyroid and hypothyroid animals. Secretion by hepatocytes from hypothyroid rats was depressed for the first 0–4 hr, but rapidly recovered. The depressed secretion of LCAT by hepatocytes from hypercholesterolemic rats correlates with the appearance in the media of apoE-rich, discoidal HDL. Discoidal HDL was six times more effective as a substrate for purified human LCAT than HDL from hypercholesterolemic serum, and twice as effective as serum and nascent HDL from euthyroid animals. It is concluded that the depressed LCAT activity in serum from hypothyroid rats is due to a depressed hepatic secretion of the enzyme and that the elevated serum activity of hypercholesterolemic rats may be related to a defect in LCAT clearance. Finally, the appearance of discoidal HDL in the medium upon culture of hepatocytes from hypercholesterolemic rats appears to be due to an inhibition of LCAT secretion by these cells. —**Ridgway, N. D., and P. J. Dolphin.** Serum activity and hepatic secretion of lecithin:cholesterol acyltransferase in experimental hypothyroidism and hypercholesterolemia. *J. Lipid Res.* 1985. **26:** 1300–1313.

Supplementary key words nascent discoidal HDL • rat hepatocytes • LCAT assay

Induction of hypercholesterolemia in the rat by administration of a diet containing 5.0% cholesterol, supplemented with 0.3% of the antithyroid agent propylthiouracil (PTU) to enhance the hypercholesterolemic effect, results in the accumulation of apoB, apoE, and apoA-I and both polar and nonpolar lipids in the serum. The accumulation of apoproteins B and E can be linked to the appearance of two abnormal lipoproteins; beta-migrating VLDL and HDL_c (1–3).

In addition, hypercholesterolemia also produces fluctuations in the activity of enzymes involved in lipoprotein metabolism in both animal models and clinical situations. The influence of the hypercholesterolemic state on lecithin:cholesterol acyltransferase (LCAT) is of particular interest due to its proposed role in reverse cholesterol transport (4). LCAT activity in the plasma of hypercholesterolemic humans and experimental animals has, in most cases, been shown to be slightly depressed or unchanged (5–8) and the mass of LCAT in the plasma of hypercholesterolemic patients has been reported to be slightly elevated (9). The most notable exception is an observation that the molar esterification rate (MER) is increased in the VLDL + LDL-depleted plasma from cholesterol-fed rabbits (10). Problems exist, however, in the interpretation of LCAT activities assayed by either the endogenous method of Stokke and Norum (11) or the common substrate method of Glomset and Wright (12), particularly in disease states when both enzyme activity and substrate availability may be altered.

Rats fed cholesterol require simultaneous induction of hypothyroidism to fully manifest hypercholesterolemia. The reasons seem to lie in reduced clearance of LDL (13) and enhanced intestinal absorption of cholesterol (14). Hypothyroidism and cholesterol feeding have some divergent effects. Most notable of these is the lack of secretion of a cholesteryl ester-rich beta-migrating VLDL by hepatocytes from hypothyroid (HT) rats. Such a lipopro-

Abbreviations: LCAT, lecithin:cholesterol acyltransferase; LDL, low density lipoprotein; VLDL, very low density lipoprotein; PTU, propylthiouracil; HT, hypothyroid; HDL, high density lipoprotein; MER, molar esterification rate; HC, hypercholesterolemia; ET, euthyroid.

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tein seems to be a de novo synthetic product of hepatocytes from hypothyroid-hypercholesterolemic (HT-HC) rats (15). Although no data are available on the combined effects of hypothyroidism and cholesterol feeding on serum LCAT activity in experimental or clinical conditions, the singular effect of hypothyroidism in both man (16) and rat (17) is to insignificantly depress enzyme levels. It is interesting to note that thyroxine treatment in rat increases enzyme activity (18), while no apparent change is evident in hypothyroid humans.

Some indirect evidence is available concerning the hepatic contribution to the serum LCAT pool in hypothyroidism and hypercholesterolemia. It has been demonstrated by us (19) that, while spherical nascent HDL accumulate in suspension cultures of HT and control rat hepatocytes, discoidal HDL reminiscent of the nascent lipoproteins described by Hamilton et al. (20) are secreted by hypercholesterolemic hepatocytes. The perfused livers of cholesterol-fed guinea pigs (21) and African green monkeys (22) also secrete a similar discoidal particle. The appearance of discoidal HDL in these instances may indicate a decreased hepatic secretion or inhibition of endogenously secreted LCAT. High cholesterol to phospholipid ratios in these nascent lipoproteins may also retard their conversion to spherical particles (23).

Here we examine the effect of hypothyroidism and cholesterol feeding on serum levels and hepatic secretion of LCAT by isolated rat hepatocytes. LCAT levels were assayed using proteoliposomes described by Chen and Albers (24), but with rat apoA-I substituted for the human apoprotein. We further examined the effectiveness of nascent HDL, secreted by hypercholesterolemic rat hepatocytes, as a substrate for purified human LCAT in light of data concerning hepatic LCAT secretion and lipoprotein composition.

MATERIALS AND METHODS

Animals

Male Long-Evans rats (Canadian Breeding Farms, St. Constant, Quebec) weighing 225–250 g were used in all experiments. Rats were made hypothyroid by administration of 0.1% propylthiouracil (PTU) in their drinking water for a period of 21–25 days. A second group of animals was fed a thrombogenic diet for 35–40 days (ICN Biochemicals, Montreal, Quebec) as previously described (15). Euthyroid animals were maintained on a standard Purina chow diet.

SDS gradient and two-dimensional polyacrylamide gel electrophoresis

Sodium dodecyl sulphate 3–15% polyacrylamide linear gradient gel electrophoresis was performed using 10-cm slab gels and a Bio-Rad model #220 vertical slab gel electrophoresis chamber as described (25). A 1-cm 3% stacking gel, pH 6.8, was employed above the pH 8.8 gradient gel. The electrophoresis buffer was 0.05 M Tris-glycine, pH 8.3, containing 0.1% SDS. Gels were run at 35 mA for 5.5 hr and stained in Coomassie Blue R250.

Two-dimensional gel electrophoresis was performed essentially as described by O'Farrell (26) using the SDS 3–15% polyacrylamide gradient gel described above for the second dimension. The first dimension isoelectric focusing employed 0.2 × 10 cm 7.5% polyacrylamide gels with pH 2.5–6.5 or pH 4.0–6.5 carrier ampholines (Pharmalytes, Pharmacia, Upsala, Sweden) as described by Gidez, Swaney, and Murnane (27). Isoelectric focusing gels were stained by the method of Righetti and Drysdale (28) and the SDS gels were stained with Coomassie Blue R250 as above.

Neuraminidase treatment of VLDL

Rat VLDL protein (0.5 mg) was incubated in 1 ml of 0.5 M sodium acetate buffer, pH 5.0, containing 0.05 mg of *Clostridium perfringens* neuraminidase (sp act 150 units/mg, Sigma Chemical Co., St. Louis, MO) for 4 hr at 35°C. Desialylation was terminated by addition of the reaction mixture to ethanol–diethyl ether 3:1 (v/v) to delipidate the VLDL prior to electrophoresis.

Preparation and culturing of hepatocytes

Rat hepatocytes were isolated using collagenase (Boehringer-Mannheim) and incubated in suspension culture for 12 or 24 hr according to the method of Jeejeebhoy et al. (29) as later modified by Krul and Dolphin (15). Isolated hepatocytes from a single liver were incubated in 300 ml of Weymouth's MB 752/1 medium (Gibco Laboratory Supplies, Grand Island, NY) containing 17.5% (v/v) horse serum (Flow Laboratories, McLean, VA). Prior to use the horse serum was delipidated by treatment with Cab-O-Sil (30) and heat-inactivated at 56°C for 45 min. This treatment effectively removed all horse serum lipoproteins and LCAT activity. Incubation of 100 μl of medium containing 17.5% delipoproteinated, heat-inactivated horse serum with proteoliposomes for 8 hr resulted in the recovery of only 200–600 cpm as cholesteryl ester. Secreted LCAT rendered activities two- to fourfold above this value after 12 hr of hepatocyte culture. The viability of the cultured hepatocytes, as determined by the leakage of the cytosolic enzyme lactate dehydrogenase, was better than 85% and 70% of initial cell viability after 12 and 24 hr, respectively.

Purification of human and rat apoA-I and preparation of proteoliposomes

Human and rat apoA-I were isolated from the HDL (d 1.063–1.21 g/ml) of plasma and serum, respectively, by

preparative isoelectric focusing following delipidation (31). After refocusing, the apoA-I from each source was found to be homogenous as determined by SDS electrophoresis in 3–15% polyacrylamide gels. Lyophylized apoA-I was redissolved in 3 M guanidine-HCl, 10 mM Tris-HCl, 0.154 M NaCl, and 1 mM EDTA, and dialyzed against the same buffer without guanidine-HCl. The guanidine-free buffer will be referred to as Tris-HCl buffer hereafter. Solubilized apoA-I was used in the preparation of proteoliposomes. Protein was determined by the method of Lowry et al. (32). Human and rat LCAT assays employed an egg yolk phosphatidylcholine, cholesterol, apoA-I liposome radiolabeled with [1,2-^{3}H(N)]cholesterol (20,000 cpm/nmol of cholesterol) (24). The molar ratio of phosphatidylcholine to cholesterol was periodically checked by gas–liquid chromatography.

Assay of rat serum and cell culture media LCAT activities

The assay mixture for rat serum LCAT contained 250 nmol of phosphatidylcholine, 12.5 nmol of cholesterol, and 1.1 nmol of rat apoA-I in the form of a proteoliposome, 0.230 ml of Tris-HCl buffer, and 0.125 ml of 2.0% fatty acid-free BSA (Sigma Chemical Company, St. Louis, MO). The mixture was vortexed and preincubated at 37°C for 20 min. At the end of this period, 0.025 ml of a 100 mM solution of 2-mercaptoethanol was added, followed by 0.015 ml of whole or VLDL + LDL-depleted serum. The mixture was vortexed and incubated at 37°C for 30 min. The reaction was terminated by the addition of 8 ml of chloroform–methanol 2:1 (v/v) and the organic phase was separated and washed by the method of Folch, Lees, and Sloane Stanley (33). The organic phase was evaporated under nitrogen and the residues were dissolved in chloroform and applied to thin layers of silica gel G. The plates were developed in heptane–isopropyl ether–acetic acid 60:40:4 (v/v/v); lipids were visualized by exposure to iodine vapors and those corresponding to cholesteryl ester and cholesterol were scraped and counted.

Hepatocytes, prepared from one liver each from control, HT, or HC-HT animals, were isolated and cultured in suspension as previously described. A time-zero blank was removed immediately after the addition of cells to the culture medium to permit subtraction of future estimates of LCAT activity from the low levels of residual activity present in the culture medium. At 1, 3, 6, 9, and 12 hr, 2-ml aliquots of culture medium were taken and the cells were removed by low speed centrifugation. LCAT activity was determined in the cell-free culture medium as follows. To 0.1 ml of the proteoliposome were added 0.130 ml of Tris-HCl buffer and 0.125 ml of 2% BSA followed by vortexing and a 20-min preincubation; 0.025 ml of 100 mM 2-mercaptoethanol and 0.100 ml of cell-free incubation medium were then added. The mixture was vortexed and incubated for 8 hr at 37°C. During this period cholesterol esterification remained essentially linear. Extraction of lipids and quantitation of cholesterol and cholesteryl ester radioactivity were as described above.

Purification of LCAT

Human plasma LCAT, to be used in incubations with whole lipoproteins, was purified according to a modified procedure described by Doi and Nishida (34). The modifications involved deletion of the second chromatography on DEAE A-50 and insertion of a final step using Con-A affinity chromatography. LCAT, purified 7,000-fold by this method, was found to contain a minor contaminant comprising approximately 5–10% of the stainable material with similar mobility to human apoA-I, as determined by SDS-PAGE in 3–15% gradient gels. **Fig. 1A** shows a 3–15% polyacrylamide gradient gel of the LCAT preparation after hydroxyl apatite chromatography. Subsequent affinity chromatography on Con-A Sepharose substantially reduces the proportion of the lower molecular weight contaminant. Two-dimensional electrophoresis of the Con-A-purified preparation (Fig. 1B) suggested that the contaminant may be apoD (mol wt 32,500, containing three isoproteins of more acidic pI than apoA-I) and not apoA-I (mol wt 28,300). Transfer of the electrophoresed proteins to nitrocellulose followed by exposure to rabbit anti-human apoD (kind gift of Dr. W. McConathy) and development with horseradish peroxidase-conjugated goat anti-rabbit gamma globulin confirmed the identity of the contaminating protein as apoD.

LCAT incubation with nascent and serum HDL

HDL cholesterol was labeled using a [^{3}H]cholesterol-albumin complex prepared according to the method of Porte and Havel (35). Incubation of LCAT with serum and nascent HDL involved preincubation of 4 μg of lipoprotein cholesterol, 250,000 cpm of [^{3}H]cholesterol-albumin complex, and enough 5.0% albumin to give a final concentration of 1.0%. This was followed by Tris-HCl buffer to give a final volume in the preincubation mixture of 0.650 ml. All incubations were carried out in 10-ml screw-top vials. The tubes were then incubated at 37°C for 30 min, at the end of which 100 mM 2-mercaptoethanol was added to a final concentration of 5 mM. This was followed by 15 units of LCAT (a unit of activity is defined as the amount of enzyme that will esterify 1 nmol of cholesterol in 1 hr using as a substrate the artificial proteoliposome described by Chen and Albers (24)), vortexing, and further incubation at 37°C. At 0, 2, and 4 hr, the reaction was terminated by the addition of 8 ml of chloroform–methanol 2:1 (v/v) and the lipids were extracted and separated as above.

Lipoprotein isolation and analysis

Lipoproteins were isolated from cell culture media and rat serum by sequential ultracentrifugation in a 50.2 Ti

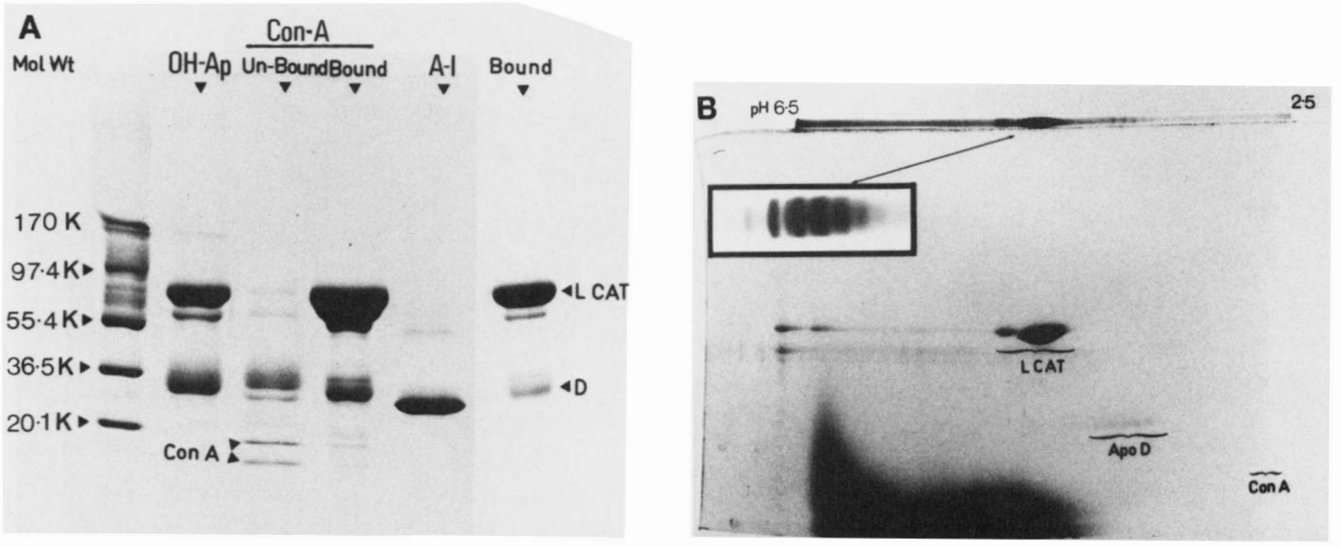

Fig. 1. Analysis of purified human LCAT by SDS 3–15% polyacrylamide gradient gel electrophoresis and two-dimensional gel electrophoresis. A, Coomassie blue-stained SDS 3–15% polyacrylamide gradient gel of the human LCAT preparation after hydroxylapatite and Con-A affinity chromatography. The contamination is substantially reduced by the use of Con-A. B, Con-A-purified LCAT preparation after analysis by two-dimensional gel electrophoresis. The first dimensional isoelectric focusing utilized a pH 2.5–6.5 gradient in a 7.5% polyacrylamide gel. The second dimensional SDS gel was as above. Note the presence of at least five LCAT isoproteins and three or four isoproteins of pI 5.0 that correspond to apoD.

fixed-angle rotor as previously described (36). VLDL was isolated at d 1.006 g/ml; VLDL + LDL at d 1.006–1.063 g/ml; and HDL at d 1.063–1.21 g/ml. The lipids of rat serum and whole lipoproteins were analyzed by gas–liquid chromatographic total lipid profiling as previously described (37). Apolipoproteins B, E, and A-I were quantitated by electroimmunoassay as described previously by us (2, 15).

Electron microscopy

Nascent lipoproteins and apoA-I proteoliposomes were negatively stained with 2% phosphotungstate, pH 7.2, on Formvar or Butvar coated 200-mesh copper grids. Grids were viewed and photographs were taken with a Philips 200 electron microscope. The resulting photographic prints were used for lipoprotein size determinations. One hundred particles were measured unless otherwise stated.

RESULTS

Assay of rat serum LCAT activities

The assay of rat LCAT in serum and cell culture media was performed using a rat apoA-I proteoliposome similar to that described by Chen and Albers (24) for the assay of the human enzyme. Liposomes, having a phospholipid to cholesterol ratio of 20:1, were prepared in the presence of different amounts of purified rat apoA-I. **Fig. 2** shows that incorporation of 1.1 nmol of rat apoA-I per assay resulted in optimal activation of rat serum LCAT. This amount of rat apoA-I was used in all further assays of rat serum and media LCAT activities. The liposome has a high capacity for cholesteryl ester as can be inferred by the linearity of the esterification rate over a period of 4 hr (**Fig. 3**). Negatively stained preparations showed the particles to be spherical in nature with a mean diameter of 140 ± 6 Å (**Fig. 4**). These dimensions are similar to those described for the liposome containing human apoA-

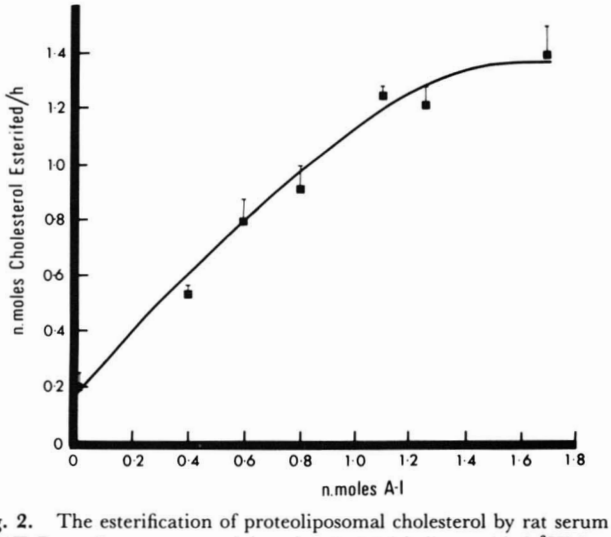

Fig. 2. The esterification of proteoliposomal cholesterol by rat serum LCAT. Proteoliposomes containing phosphatidylcholine and [1,2-^{3}H]cholesterol in a 20:1 molar ratio were prepared with the indicated quantity of associated rat apoA-I. The proteoliposomes were then incubated with 15 μl of euthyroid rat serum as a source of LCAT. The incubation conditions and subsequent determination of cholesteryl ester formation were as described in Methods. The results are expressed as the mean of four experiments ± SEM.

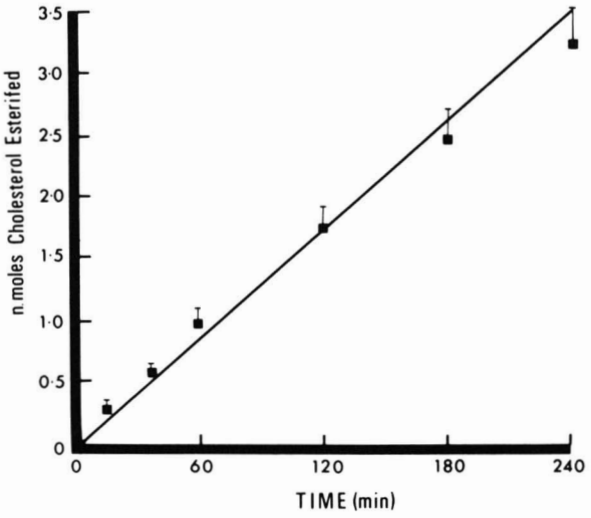

Fig. 3. Time course of cholesterol esterification in rat apoA-I proteoliposomes using euthyroid rat serum as a source of LCAT. The conditions for incubation and determination of cholesteryl ester formation were as described in Methods. Each value represents the mean ± SEM of four experiments.

I (24). Proteoliposomes prepared in this manner contained 5–8% of discoidal material as determined by electron microscopy.

Several laboratories have presented evidence that suggests that estimates of serum LCAT activity using artificial, exogenous substrates should be free from the effects of any inhibitory factors in serum, particularly when small volumes of serum are used (24, 34). **Fig. 5** shows the dependence of cholesterol esterification on the volume of normal rat serum when esterification rates were calculated with and without taking into account the contribution of serum cholesterol. It is evident that cholesterol esterification does not remain linear up to 15 μl serum volumes in either case. However the linearity is somewhat better when calculations include serum cholesterol. It has been demonstrated that cholesterol will exchange rapidly between HDL and phospholipid-cholesterol liposomes (38) and between HDL and LDL (39). In the former instance exchange was concentration-dependent. Significant amounts of lipoprotein cholesterol added to the assay, in the form of lipoprotein, will permit cholesterol in the pool of liposomes to exchange with this lipoprotein cholesterol and result in a redistribution of label between lipoproteins and liposomes. If, at the same time, the extent of LCAT-catalyzed esterification of cholesterol varies from lipoprotein to lipoprotein and between lipoprotein and liposome, the result will be an over- or underestimation of enzyme activity. The problem of isotope exchange and differences in substrate efficiency are further compounded in disease states in which lipoprotein composition and concentration may be markedly altered. **Table 1** compares serum LCAT activities of euthyroid, hypothyroid, and hypercholesterolemic rats. It should be noted that the latter two conditions produce marked fluctuations in serum lipoprotein and cholesterol levels (2, 40). Calculations of esterification rates based solely on liposomal cholesterol revealed that both hypothyroid and hypercholesterolemic serum LCAT activities are one-half the control MER of 0.82 ± 0.05. Estimations of LCAT activities based on serum and liposomal cholesterol are markedly different; hypercholesterolemic serum activities are elevated approximately fourfold relative to both euthyroid and hypothyroid serum activities. Both of these estimates of serum LCAT activities are probably erroneous due to isotope exchange and substrate variabilities among the cholesterol-containing particles within a given assay. We feel the best estimates of LCAT activities are gained by adjusting the density of a sample of plasma to 1.063 g/ml, centrifuging for an allotted period of time, and assaying the VLDL + LDL-free infranatant. This procedure removes 80–95% of the serum cholesterol (Table 1). Hypercholesterolemic rat serum activity in the $d > 1.063$ g/ml fraction is 1.56 ± 0.14 compared to the MER of 0.96 ± 0.06 in serum from euthyroid rats. In contrast, activity in the same fraction from hypothyroid

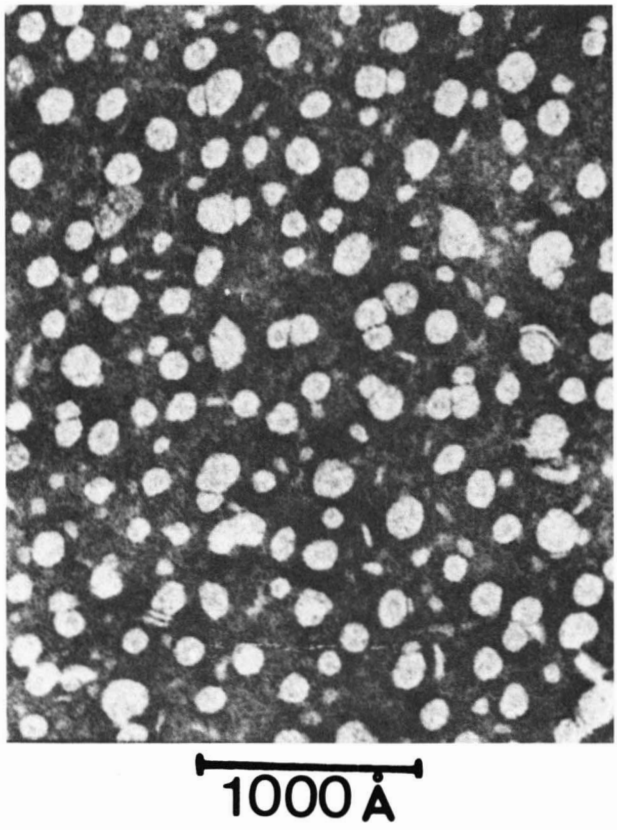

Fig. 4. Electron micrograph of negatively stained preparations of rat apoA-I proteoliposomes. Magnification 290,000 diameters. The liposomes have a mean diameter of 140 ± 6 Å (n = 100). Discoidal material represents 5–8% of the total.

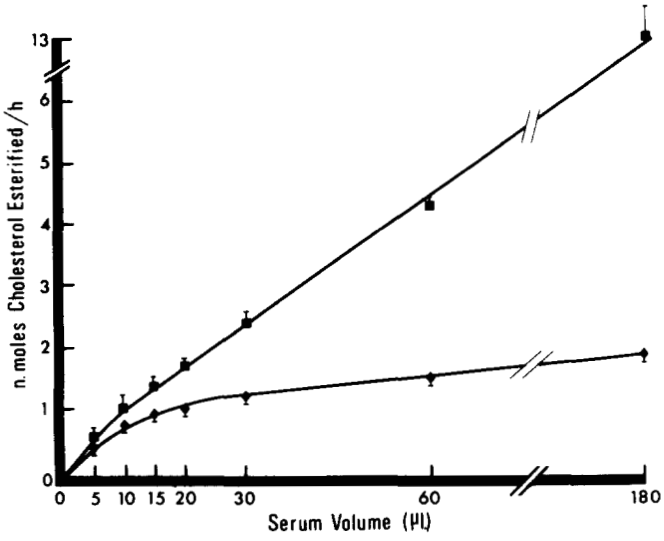

Fig. 5. The dependence of proteoliposomal cholesterol esterification upon serum volume. (■), Molar esterification rate (MER) calculated including serum cholesterol; (♦), MER calculated excluding serum cholesterol.

rats is about one-half of control values. It should be noted that removal of the majority (76%) of the cholesterol from euthyroid rat sera had no significant effect upon the LCAT activity as measured with proteoliposomes (MER = 0.82 ± 0.05 for whole sera vs. 0.94 ± 0.06 for the d > 1.063 g/ml fraction). Further reduction of the serum cholesterol by ultracentrifugation at d 1.21 g/ml was considered impractical as this would remove the majority of the serum LCAT activity in association with the HDL (9, 41).

Hepatic secretion of LCAT

The only organ known to synthesize and secrete LCAT is the liver (42, 43). This organ is also thought to play a role in the clearance of the enzyme from plasma (44), although the exact mechanisms remain obscure. **Fig. 6** shows the secretion and accumulation of LCAT in media in which hepatocytes from euthyroid, hypothyroid, and hypercholesterolemic rats were cultured. It is evident that secretion of LCAT by isolated hypercholesterolemic hepatocytes is significantly depressed relative to controls and hypothyroid hepatocytes at 12 hr. Examination of secretion earlier in the time course (0–4 hr) shows that both hypercholesterolemic and hypothyroid hepatocytes secrete less LCAT than controls.

Hepatic secretion of lipids

The lipid secretion rates determined after 12 hr of culture for ET and HC-HT cells are shown in **Table 2.** With the possible exception of d 1.063–1.21 g/ml triacylglycerols, HC-HT cells secrete significantly more of each lipid component into both VLDL + LDL and HDL density ranges than do euthyroid hepatocytes. This confirms our previous observations (15) and is particularly significant with respect to cholesterol inasmuch as, in the assay of secreted LCAT, isotope dilution similar to that noted for serum may have accounted for the observed diminution in secretion by HT-HC hepatocytes. Determination of the culture media cholesterol content after 12 hr of incubation showed that 100 μl of medium from HT-HC hepatocyte incubations contained 0.94 μg of cholesterol, while 100 μl of medium from euthyroid hepatocytes contained 0.64 μg. Inspection of the data in Table 1 shows that 15 μl of euthyroid rat serum used in the assay of LCAT contains 2.16 μg of cholesterol and 15 μl of the d > 1.063 g/ml fraction contains 0.52 μg of cholesterol. Assay of each of these fractions yielded estimates of LCAT activity which were not significantly different (0.82 ± 0.05 vs 0.94 ± 0.06 nmol of cholesterol esterified per hr) as noted earlier. As the mass of cholesterol present within the 100 μl of culture media assayed falls within this range, isotope dilution would not contribute significantly to the observed results for media LCAT activity when assayed under these conditions. Thus, elevated secretion of cholesterol of HC-HT cells could not account for the

TABLE 1. LCAT activities in sera from euthyroid, hypothyroid, and hypothyroid-hypercholesterolemic rats

	Liposomal Cholesterol	Serum Cholesterol	Liposomal + Serum Cholesterol	d 1.063 g/ml Cholesterol	d > 1.063 g/ml Cholesterol
	nmol esterified/hr	*mg/dl*	*nmol esterified/hr*	*nmol esterified/hr*	*mg/dl*
Euthyroid	0.82 ± 0.05	14.4 ± 1.6	1.14 ± 0.08	0.94 ± 0.06	3.46 ± 0.97
Hypothyroid	0.45 ± 0.06[a]	25.4 ± 2.9	0.96 ± 0.08	0.67 ± 0.05[b]	5.27 ± 1.49
Hypothyroid-hyperchol.	0.43 ± 0.05[a]	263.5 ± 83.9	4.30 ± 1.40[c]	1.56 ± 0.14[a]	17.37 ± 5.37
N	7	5	5	5	5

Each value represents the mean ± SEM of the indicated number (N) of determinations. Serum cholesterol levels were determined by gas–liquid chromatographic total lipid profiling (37). Significant differences from the euthyroid values as estimated by Student's *t*-test (two-tailed) are shown for the following probabilities: [a], $P < 0.001$; [b], $P < 0.01$; [c], $P < 0.05$.

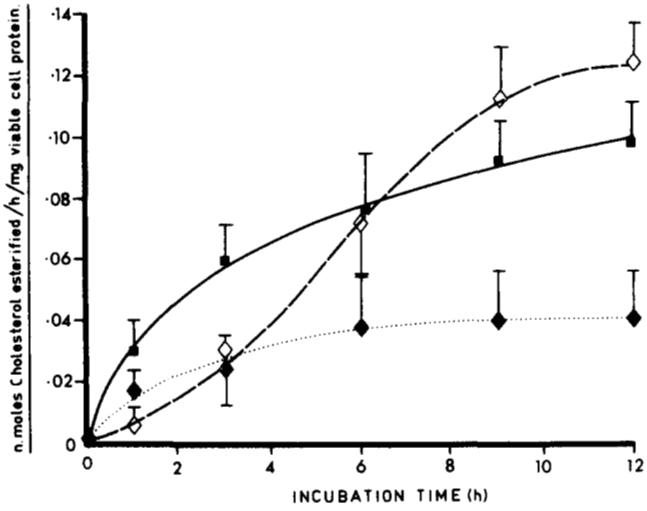

Fig. 6. The secretion of LCAT activity by isolated rat hepatocytes. Isolated rat hepatocytes were prepared and incubated as described in Methods. Aliquots of the media were removed at the indicated times and the cell-free supernatant was assayed for LCAT activity using rat apoA-I proteoliposomes. (■), Euthyroid hepatocytes, n = 5; (◇), hypothyroid hepatocytes, n = 3; (◆), hypothyroid-hypercholesterolemic hepatocytes, n = 4. LCAT activity in the media is expressed in terms of viable cell protein as determined by the leakage of LDH. Each value represents the mean ± SEM for the indicated number of experiments.

observed, pronounced decrease in media LCAT activity in these incubations.

Degradation of secreted LCAT by residual collagenase

Preparation of hepatocytes from rat liver utilizes commercial preparations of collagenase which vary in their content of proteolytic enzymes. Conceivably these enzymes could be transferred to the culture medium and result in proteolysis of nascent secreted proteins including LCAT. This would result in an underestimation of LCAT activity and secretion rate. Capuzzi, Sparks, and DeHoff (45) reported such a proteolytic cleavage of the C apolipoproteins secreted by hepatocytes cultured in a serum-free medium. In order to examine this possibility, different concentrations of tissue dissociation-grade collagenase were tested for their ability to inactivate rat serum LCAT (**Fig. 7**). Fifteen μl of rat serum was incubated for 4 hr in Weymouths media in the presence or absence of 17.5% delipidated horse serum and with various concentrations of collagenase. The highest of these concentrations (200 μg/ml) was used in the isolation of hepatocytes and, due to the number of washes of cells after isolation, would not approach the amount of residual collagenase present in the culture media. After incubation LCAT activity was determined. At increasing concentrations of collagenase, LCAT was inactivated in both instances, but to a much greater extent in the media not containing horse serum. Thus the inclusion of 17.5% horse serum in the culture medium seems to buffer LCAT from the potential effects of residual collagenase co-isolated with the hepatocytes. Similar experiments using conditioned media (medium obtained from 12-hr incubations of HT-HC hepatocytes) containing 17.5% horse serum showed no inactivation of rat serum LCAT over a 4-hr period (results not shown).

Influence of LCAT on plasma and nascent HDL

The lipid composition of nascent lipoproteins secreted by euthyroid and hypercholesterolemic hepatocytes is shown in **Table 3.** Hypercholesterolemic HDL is enriched in cholesterol and depleted in triglyceride relative to euthyroid nascent HDL. Euthyroid nascent HDL, which appears as a spherical particle of mean diameter 168.2 ± 1.6 Å under the electron microscope (**Fig. 8**), probably represents a particle that has been acted upon by LCAT in the incubation medium. Euthyroid nascent HDL has 50% of its lipid mass as surface lipids, compared to 70% in the HC nascent HDL, indicating that

TABLE 2. Lipid secretion rates of hepatocytes from hypothyroid-hypercholesterolemic and euthyroid rats in suspension culture

	Hypothyroid-Hypercholesterolemic		Euthyroid	
Lipid	1.006–1.063 g/ml	1.063–1.21 g/ml	1.006–1.063 g/ml	1.063–1.21 g/ml
	μg per hr per g cell protein			
Triacylglycerols	252.1 ± 34.0[a]	14.0 ± 4.7	127.2 ± 5.7	9.0 ± 6.5
Cholesterol	84.1 ± 22.6[a]	13.9 ± 2.8[b]	8.2 ± 2.6	1.9 ± 0.8
Cholesteryl esters	349.5 ± 180.3[c]	10.6 ± 4.4[c]	18.4 ± 8.6	2.5 ± 1.9
Phospholipids	160.9 ± 37.8[d]	35.9 ± 10.5[d]	57.0 ± 13.9	5.4 ± 1.6
Total lipid	837.4 ± 241.9[d]	74.5 ± 21.7[d]	260.6 ± 84.2	18.9 ± 10.8

Hepatocytes were prepared from each group of rats and placed in suspension culture for 24 hr as described in Methods. Cells were then removed by low speed centrifugation, the lipoproteins were fractionated by ultracentrifugation, and total lipids were analyzed by gas–liquid chromatographic total lipid profiling (37). Each value represents the mean ± SEM for three determinations. Significance of difference from the euthyroid values as estimated by Student's *t*-test (two-tailed) is shown for the following probabilities: [a], $P < 0.02$; [b], $P < 0.01$; [c], $P < 0.1$; [d], $P < 0.05$. With the possible exception of the HC/HT d 1.063–1.21 g/ml triacylglycerols, HC/HT hepatocytes secrete elevated quantities of all lipid components into both fractions when compared to ET control cells.

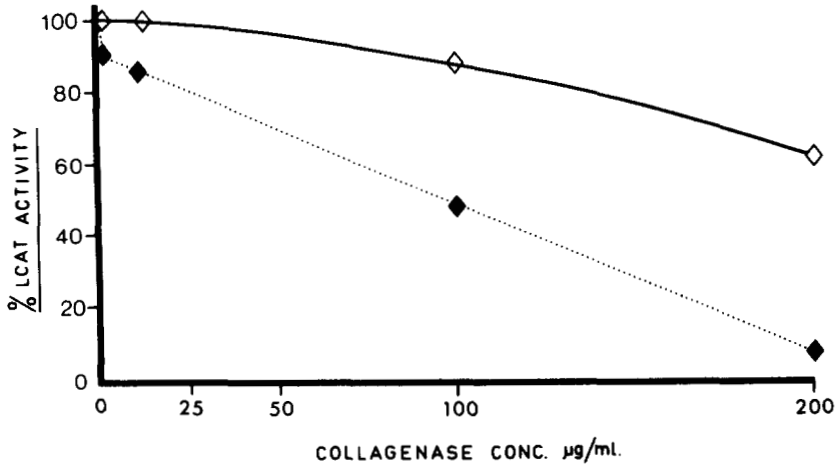

Fig. 7. The inactivation of rat serum LCAT by collagenase in the presence and absence of horse serum. Fifteen µl of euthyroid rat serum (used as a source of LCAT) was incubated for 4 hr in Weymouth's medium with and without horse serum at a final concentration of 17.5%, and in the presence of the indicated concentration of collagenase. Following incubation, the residual LCAT activity was assayed as described in Methods. (◊) Weymouth's plus 17.5% horse serum; (♦) Weymouth's minus 17.5% horse serum. Each value represents the average of two determinations.

the former represents a partially catabolized particle. The ratio of cholesterol to cholesteryl esters (C/CE) in ET HDL is 0.7, whereas that of nascent HC-HT HDL is significantly higher at 1.25. These data are consistent with reduced LCAT secretion by HC-HT cells and the presence of discoidal lipoproteins. The C/CE ratios of secreted VLDL + LDL for ET and HC-HT hepatocytes are 0.47 and 0.26, respectively. As cholesteryl esters in these particles are derived primarily through the action of hepatic ACAT, the data reflect the observed elevated secretion of cholesteryl esters by the HC-HT cells. The lower apoB to apoE ratio and almost complete absence of apoA-I in the HC-HT nascent HDL (**Table 4**) is indicative of large apoE-rich discs that have been described elsewhere (20, 46–51). Indeed, negatively stained preparations of nascent HT-HC HDL contain discoidal particles with diameters of 220 Å and widths of 40–50 Å (**Fig. 9**). The spherical lipoproteins seen in Fig. 9 have diameters between 90 and 166 Å, which is within the range seen in Fig. 8 for the HDL particles isolated from suspension culture of euthyroid hepatocytes. Thus the spherical particles present in the HDL fraction from HC-HT cell cultures may represent nascent HDL that has been transformed into spherical particles by the action of the limited quantity of LCAT secreted by these cells.

The relative absence of LCAT activity in the media in which HC-HT hepatocytes have been cultured is the first direct evidence to suggest why discoidal HDL accumulate in this instance. If the discoidal HDL accumulation is due to an enzyme deficiency alone, it would follow that the nascent HDL would be a good substrate for LCAT. To test this possibility, the viability of nascent discoidal HDL as substrates for partially purified human LCAT was tested. **Fig. 10** shows the extent of LCAT-catalyzed esterification

TABLE 3. Lipid composition of nascent lipoproteins secreted by hepatocytes from euthyroid and hypothyroid-hypercholesterolemic rats

	Hypothyroid-Hypercholesterolemic		Euthyroid	
Lipid	d 1.006–1.063 g/ml	d 1.063–1.21 g/ml	d 1.006–1.063 g/ml	d 1.063–1.21 g/ml
Triacylglycerols	42.8 ± 3.5[a]	13.8 ± 2.9[b]	70.3 ± 3.8	38.1 ± 12.6
Cholesterol	7.4 ± 0.9[c]	19.8 ± 1.4[a]	2.9 ± 0.6	10.9 ± 1.0
Cholesteryl esters	28.9 ± 2.2[a]	15.9 ± 2.1	6.2 ± 1.4	14.0 ± 2.4
Phospholipids	20.8 ± 0.8	50.5 ± 1.6	20.6 ± 2.4	37.1 ± 12.6
C/PL	0.75 ± 0.10[c]	0.83 ± 0.06	0.30 ± 0.05	0.72 ± 0.12
N	5	4	7	6

Lipid analysis of freshly isolated lipoproteins obtained after 24 hr of culture was performed by gas-liquid chromatographic total lipid profiling (37). Each value represents the mean ± SEM for the indicated number (N) of determinations. Significance of difference from the euthyroid values as estimated by Student's *t*-test (two-tailed) is shown for the following probabilities: [a], $P < 0.001$; [b], $P < 0.05$; [c], $P < 0.01$.

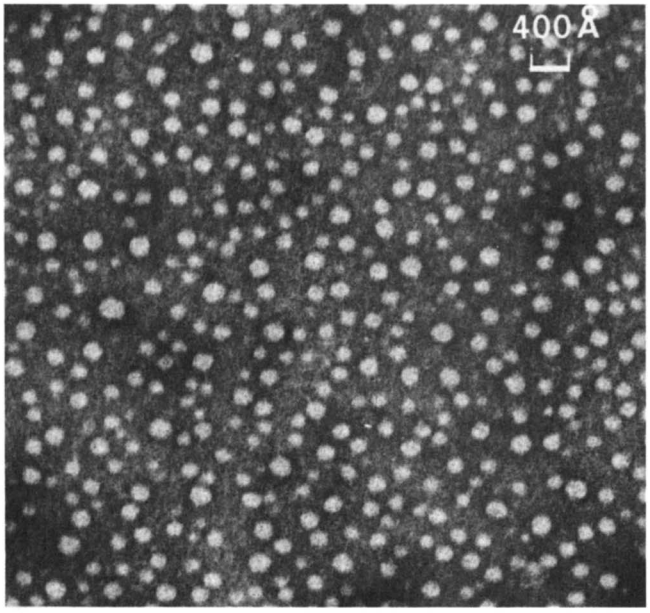

Fig. 8. Electron micrograph of nascent HDL from incubations of hepatocytes from euthyroid rats. Magnification 136,300 diameters. The HDL was isolated at d 1.063–1.21 g/ml, and negative-stained with 2% phosphotungstate as described in Methods. The spherical particles have a mean diameter of 168.2 ± 1.6 Å (n = 200). No discoidal lipoproteins were observed in these preparations.

of cholesterol in nascent and serum HDL as determined radiochemically. Cholesterol in HC nascent discoidal HDL was esterified to a sixfold greater extent than its serum counterpart and 2.5 times more efficiently than euthyroid serum HDL. The substrate efficiency of euthyroid nascent HDL is similar to that reported by Hamilton et al. (20), again indicating that it has already been acted upon by endogenously secreted LCAT.

Effects of the induction of hypercholesterolemia and hypothyroidism upon the serum apoE isoprotein pattern

In addition to causing a marked increment in serum LCAT with decreased hepatic secretion, induction of the HC-HT state also results in marked effects upon the serum apoE isoprotein pattern. **Fig. 11** shows the two-dimensional gel electrophoretic profile of apoE isolated from a variety of lipoproteins from ET and HC-HT rats. ApoE in nascent VLDL + LDL from both ET and HC-HT rat hepatocytes contains predominantly isoproteins 1 and 3. Little or no C apoproteins were associated with these nascent lipoproteins. The two major apoE isoproteins in serum VLDL and HDL from ET rats, in contrast, are isoproteins 1 and 2. Inspection of the apoE profiles of VLDL and LDL from HC-HT rat sera show a significantly elevated proportion of the more acidic isoproteins (3, 4) when compared to euthyroid lipoproteins. Furthermore, isoproteins 1 and 3 predominate as in the nascent lipoproteins from the cultured hepatocytes. **Fig. 12** shows that treatment of ET serum VLDL with neuraminidase virtually eliminates isoproteins 2, 3, and 4 from the gel and indicates that the observed heterogeneity of apoE upon isoelectric focusing is largely due to increasing sialic acid content. These data indicate that the induction of the HC-HT state results in the accumulation of more highly sialylated forms of apoE within the plasma lipoproteins, and that the normal conversion of isoprotein 3 into isoprotein 2 and 1 by removal of sialic acid may be defective. Heterogeneity of the apoE pattern in the second, SDS, dimension may be attributable to artifacts resulting from the generation of ampholine-protein complexes during first dimension electrophoresis.

DISCUSSION

In this report we have extended our studies on the metabolism and hepatic synthesis of lipoproteins in experimental hypothyroidism and hypercholesterolemia to include effects upon the plasma enzyme LCAT. Increased dietary cholesterol and the resulting elevation in plasma and tissue cholesterol levels would be expected to tax the reverse cholesterol transport route and, in particular, LCAT. The secretion of LCAT by isolated hepatocytes was determined in response to treatment of rats with PTU and cholesterol feeding. Hepatocyte secretion was then related to the level of enzyme in the serum of whole, identically treated, animals and to the appearance of discoidal lipoproteins within the hepatocyte incubation media.

Hypothyroidism and hypercholesterolemia have divergent effects on the serum activity of LCAT: the former resulting in an approximate 30% decrease in activity and the latter treatment elevating enzyme activity significantly above control values. These data indicate that in HC-HT rats it is metabolic perturbations due to cholesterol feeding that elevate serum LCAT levels. Our

TABLE 4. The ratio of apoB to apoE in nascent lipoproteins

	Fraction	
Hepatocytes	d 1.006–1.063 g/ml	d 1.063–1.21 g/ml
HC-HT	2.91 ± 0.46 (6)	0.50 ± 0.19 (4)
ET	4.31 ± 0.58 (5)	4.75 (2)

Apolipoproteins were quantitated by electroimmunoassay as described (2, 15). HC-HT, hypothyroid-hypercholesterolemic; ET, euthyroid. Each value represents the mean ± SEM for the number of determinations in parentheses. Only trace quantities of apoA-I could be detected in the d 1.006–1.063 g/ml or d 1.063–1.21 g/ml fraction from either group of hepatocytes. These data show that the HDL secreted by HC-HT hepatocytes is very rich in apoE and contains little or no apoA-I. The presence of apoB in the HDL density range of hepatocyte culture media has also been noted by others (59).

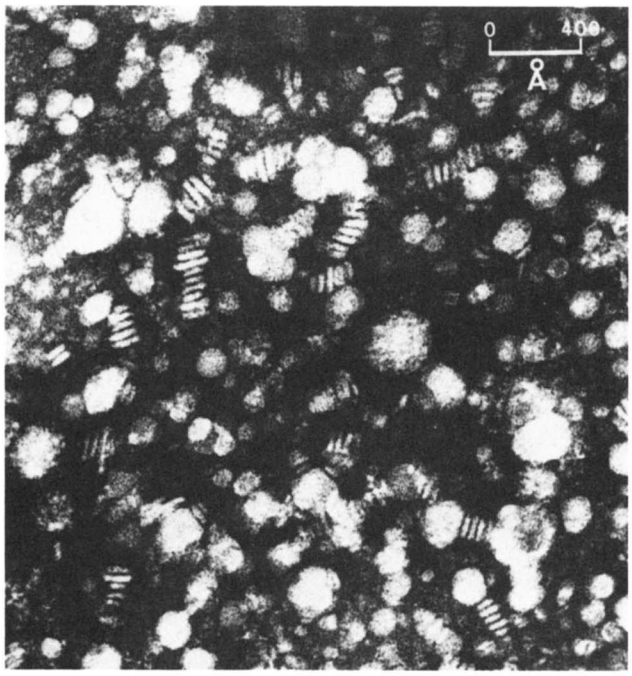

Fig. 9. Electron micrograph of nascent HDL from incubations of hepatocytes from hypothyroid-hypercholesterolemic rats. Magnification 300,000 diameters. HDL was isolated at d 1.063–1.21 g/ml. The discoidal particles have a mean diameter of 220 Å and width of 40–50 Å. The smaller number of spherical particles have diameters between 90 and 166 Å and may represent discoidal material transformed into spherical particles by the low levels of LCAT present in the culture medium.

observations on serum LCAT activities are in some disagreement with values reported in the literature. Rosenqvist, Mahler, and Carlsson (17) noted that treatment of rats with the thyrostatic agent 1-methyl-2-mercaptoimidazole for periods up to 4 weeks gave a statistically insignificant depression of serum LCAT activity. Treatment with this agent produced changes in lipoprotein patterns and lipoprotein lipase activity similar to those reported elsewhere. The same report (17) showed an increase in LCAT activity upon treatment with thyroxine, however. Measurement of plasma LCAT in hypothyroid human subjects has revealed an unchanged (52) or slightly depressed (16) activity. Unlike the rat, no elevation in LCAT was evident upon treatment of hypothyroid patients with thyroxine (16). Cholesterol feeding in the rat and rabbit have shown LCAT activity to be either reduced (7, 8) or unchanged (10) in whole plasma, respectively. It is interesting to note that in the study performed by Pinon and Laudat (10), LCAT activity was found to be elevated in plasma depleted in VLDL and LDL by either ultracentrifugation or heparin-magnesium chloride precipitation. These LCAT activities, assayed by the method of Stokke and Norum (11), are similar to our results when one compares plasma and the d > 1.063 g/ml fraction of plasma from cholesterol-fed rats.

Comparison of plasma and VLDL + LDL-depleted plasma LCAT activities raises some important points concerning the assay of this enzyme using artificial substrates. The original "self" method of LCAT assay (11) measured in vivo esterification rates and not actual enzyme mass, as is proposed for the artificial exogenous substrate systems and the common substrate methods (12, 24). The assumption that assays employing artificial substrates determine actual active enzyme mass may however fail under some conditions. The most noteworthy of these pitfalls is encountered upon assay of serum in which cholesterol concentrations are elevated. The rapid equilibration of labeled cholesterol between lipoproteins present in the serum and the phosphatidylcholine-cholesterol liposomes makes the interpretation of activities difficult, particularly when the lipoproteins present in the assay may vary. The only clear way to reconcile this problem is either to measure enzyme mass by immunoassay (9) or to remove the majority of cholesterol from plasma, thus alleviating the exchange or inhibition phenomena. Some workers have shown that the use of smaller volumes of serum (2 μl) may sufficiently dilute out any inhibitory factors (34). This may not be effective in light of the very

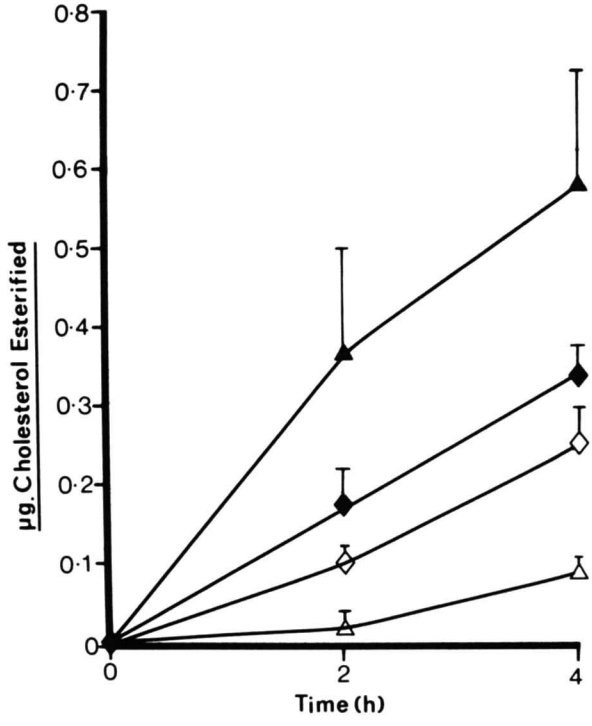

Fig. 10. The LCAT-catalyzed esterification of nascent and serum HDL cholesterol. Four μg of HDL cholesterol, prelabeled with [^{3}H]cholesterol, was incubated with purified human LCAT and the extent of cholesterol esterification was determined as described in Methods. Values represent the mean ± SEM for three experiments. Each assay was performed in duplicate. (▲), Nascent discoidal HDL from hypothyroid-hypercholesterolemic hepatocytes; (♦), nascent HDL from euthyroid hepatocytes; (◇), euthyroid serum HDL; (△), serum HDL from hypothyroid-hypercholesterolemic rats.

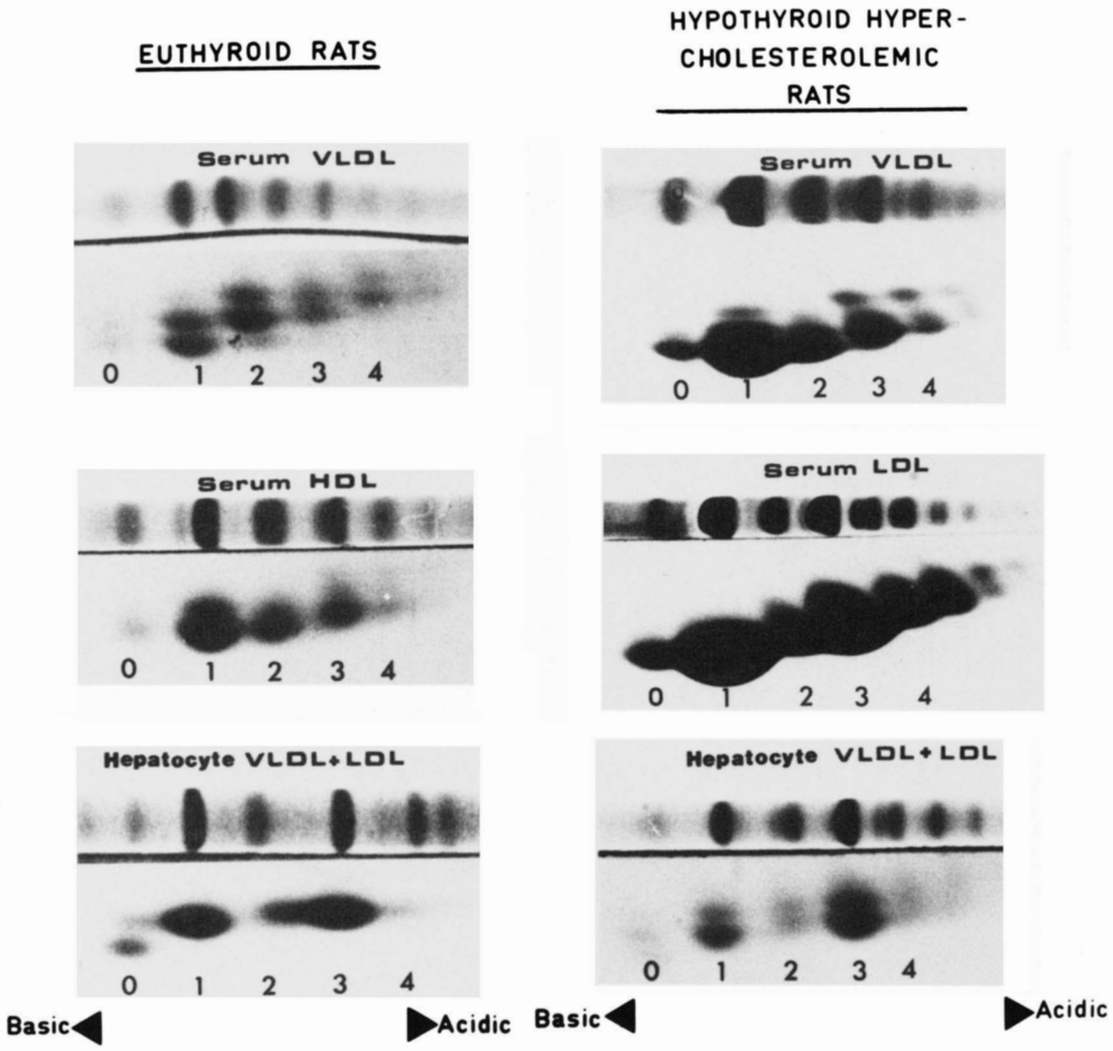

Fig. 11. Two-dimensional polyacrylamide gel electrophoresis of apoE isoproteins present in serum and nascent lipoproteins from euthyroid and hypercholesterolemic-hypothyroid rats. Lipoproteins were isolated, delipidated, and electrophoresed as described in Methods. The first dimension was isoelectric focusing (IEF) and a 7.5% polyacrylamide gel with a pH 4.0–6.5 gradient. The portion of the IEF gel containing the apoE isoproteins is shown at the top of each panel. The second dimension was a 3–15% polyacrylamide linear gradient gel containing sodium dodecyl sulfate. The portion of the SDS gel containing the apoE isoproteins is shown in the lower half of each panel. Isoproteins are designated arbitrarily from 0 to 4 and the numerical values do not necessarily reflect sialic acid content. Note the preponderance of isoproteins 1 and 3 in the nascent VLDL from both hepatocyte cultures and in the serum VLDL and LDL from HC-HT animals. Isoprotein 1 is the major form in serum VLDL and HDL from ET rat serum. The homogeneity noted in the second dimension may be attributable to artifacts generated by ampholine-protein complexes formed during isoelectric focusing in the first dimension.

high cholesterol concentrations in the serum of some cholesterol-fed animals.

The low LCAT activity in the serum of HT rats is reflected to some extent by the lag in secretion of this enzyme by the HT hepatocytes in the first 3–4 hr of culture. The low serum activities may reflect a hepatic impairment in enzyme synthesis and secretion in the early stages of culturing. From 4 to 12 hr the HT hepatocytes overcome the thyrotoxic effect of PTU, and LCAT secretion approaches and exceeds control values. The low secretion of LCAT by HC-HT hepatocytes over the entire 12-hr culturing period suggests that, while hypothyroidism should have maintained this effect for several hours, it is factors associated with cholesterol feeding that are preemptive. Elevated plasma levels of LCAT in the hypercholesterolemic rat coupled with depressed secretion of the enzyme are indicative of an impairment in LCAT metabolism at the level of enzyme removal. While the liver has been implicated in LCAT uptake (44), the exact mechanism(s) are yet to be delineated. The high sialic acid content and the absence of enzyme containing less than nine or ten sialic acid residues in the plasma (53) may indicate that desialylation within the plasma compartment leads to removal via a hepatic asialoglycoprotein receptor (54). A defect in the desialylation of LCAT in the plasma would delay its removal and result in in-

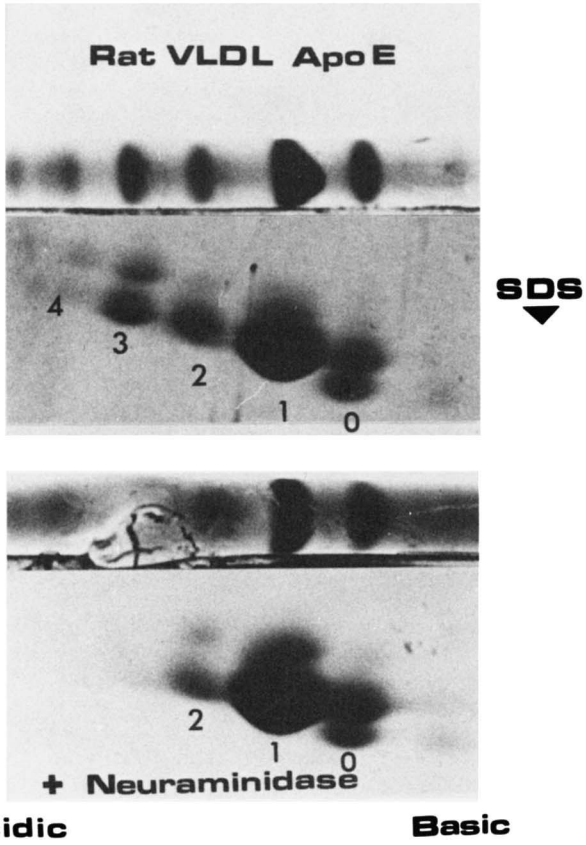

Fig. 12. Two-dimensional polyacrylamide gel electrophoresis of rat serum VLDL apoE isoproteins before and after treatment with *Clostridium perfringens* neuraminidase. Rat serum VLDL was incubated with *C. perfringens* neuraminidase as described in Methods. The VLDL was delipidated and electrophoresed as in Fig. 11. Note the absence of isoproteins 3 and 4 and the reduction in isoprotein 2 after neuraminidase treatment, indicating that the heterogeneity of apoE in the IEF dimension is due predominantly to differing sialic acid content.

creased plasma activity. The sialic acid content of apoE has been shown to increase upon cholesterol feeding in the guinea pig (55). Data shown here (Fig. 11) have also indicated that serum apoE from HC-HT rats is more highly sialylated than in euthyroid normocholesterolemic control animals. These observations are consistent with the possibility that a desialylation defect may exist upon the induction of hypercholesterolemia.

The decreased in vitro secretion of LCAT, observed in HT-HC hepatocytes, is the first evidence to suggest that nascent discoidal HDL accumulate under these conditions due to an enzyme deficiency. Similar discoidal particles have been isolated in cases of LCAT deficiency associated with cholestasis (46, 47), hereditary LCAT deficiency (48–50), and *d*-galactosamine-induced liver injury (56). The discoidal HDL isolated in these instances is rich in cholesterol and phospholipid and has apoE as the major apolipoprotein component. These lipoproteins are thought to represent the nascent hepatic secretory HDL that, upon entry into the circulation, are converted to spherical particles by the action of LCAT (20). Similarly, the nascent HDL described in this study are rich in cholesterol and phospholipid and contain apoE as the major apoprotein component. This observation, coupled with a size and morphology similar to HDL isolated from rat liver perfusates containing DTNB, would suggest that HC-HT HDL represent the primary secretory product of the liver.

Previously it has been demonstrated that apoE-rich discoidal HDL, isolated from the plasma of LCAT-deficient patients (57), peripheral lymph of cholesterol-fed dogs (51), and rat liver perfusates containing DTNB (20) are converted to spherical HDL by the action of purified LCAT. Endogenous LCAT activity in these instances is either very low or nonexistent. The effectiveness of nascent HT-HC HDL as substrates for LCAT lends further credence to the possibility that their accumulation is due to a defect in LCAT secretion. The inhibition of LCAT secretion itself may be due to the high cholesterol diet, although the effects of hypothyroidism cannot, as yet, be ruled out. Feeding of cholesterol-supplemented diets to guinea pigs (21) and African green monkeys (22) also results in the appearance of discoidal HDL in liver perfusates, although no information concerning a correlation with LCAT secretion is yet available for these species.

Discoidal HDL appear not only in the cholesterol-fed state, but also in liver perfusates from control, chow-fed guinea pigs and monkeys. While this may represent a diluting out of enzyme or substrate HDL, the possibility exists that the enzyme is being inactivated in the perfusate. This is not the case in ET cultured rat hepatocyte suspensions where nascent HDL appear in the media as spherical particles (15). It is possible that the inclusion of 17.5% delipoproteinated horse serum in all of our hepatocyte culture media protects the secreted LCAT from digestion by residual collagenase or proteases released from leaky cells during culture. LCAT activity has been detected in liver perfusates from normal rats (43) and rabbits (58), indicating that the requirements for activating factors or the lability of the enzyme may vary from species to species.

Although the primary reason for the appearance of discoidal HDL in HC-HT hepatocyte culture media may be a reduced secretion of LCAT, other factors associated with cholesterol feeding may play a role. The small amount of LCAT activity secreted by the HC-HT hepatocytes may not be able to cope with the elevated secretion of lipid and apoE into the HDL density range (15). In such an instance the enzyme would effectively be diluted out. Trace amounts of apoA-I, the primary activator of LCAT (23), associated with nascent HDL have suggested that, based upon co-factor requirements, these HDL should be poor substrates for LCAT. As noted earlier these discs are rapidly converted to spherical particles by

the action of LCAT in vitro. In vitro radiolabeling experiments not reported here failed to show the presence of significant de novo synthesized apoA-I in association with HDL isolated from ET or HC-HT hepatocyte culture media. This is consistent with our previous report (15) that the majority of the apoA-I secreted by these cells is present in a low molecular weight, virtually lipid-free form. Despite the high capacity and avid binding of Cab-O-Sil to lipoproteins and apoproteins present in the d > 1.21 g/ml fraction (59), there remained the possibility that residual horse apoA-I present within the horse serum may associate with the nascent HDL and stimulate LCAT activity towards these particles. This possibility was not evaluated here due to our lack of immunoassays for horse apolipoproteins. It should be noted, however, that the same degree of possible horse serum apoA-I contamination would be present in incubations of hepatocytes from all three animal groups and thus the possible effects would tend to cancel each other. Acquisition of plasma apoA-I in vivo and the elevated plasma LCAT activity may similarly explain the lack of discoidal HDL in the plasma of HC-HT rats, although a recent report (60) has demonstrated that two isoforms of apoE (E-2 and E-3) activate LCAT 35–40% as effectively as apoA-I. This suggests that apoE is a physiological activator of LCAT and explains, in part, the rapid conversion of apoE-rich discoidal HDL into spherical HDL in vivo and in vitro.

This work was supported by grant number MA 5999 to PJD from the Medical Research Council of Canada. We are indebted to the Canadian Red Cross for supplying fresh rejected whole blood from which human LCAT was purified. NR held a graduate student research traineeship from the Nova Scotia Heart Foundation and was the recipient of the 1985 Bristol-Myers Junior Award for Excellence in Lipoprotein Research.

Manuscript received 13 December 1984.

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