special report

Hepatic Lipoprotein and Cholesterol Metabolism. Report of a Conference

Roger A. Davis

Hepatobiliary Research Center, University of Colorado Health Sciences Center, Denver, CO 80262

The Aspen Bile Acid/Cholesterol Conference was organized to bring together scientists (both established and in training) to discuss the role of the liver in regulating plasma lipoprotein metabolism. Roger Davis, the 1987 conference chairman, summarized some of the controversies to which this year's conference was directed. It is well recognized that hepatic uptake of cholesterol and its catabolism to bile acids regulate the major pathway through which cholesterol is eliminated from the body: excretion in the form of biliary cholesterol and bile salts. Because of the prominent role of the liver in determining cholesterol homeostasis, atherosclerosis is, to a degree, a "liver disease" of the heart. In spite of great progress that has been made in understanding the structure and functions of lipoproteins, regulation of extrahepatic lipoprotein uptake and regulation of cholesterol synthesis, there are major voids in our understanding of hepatic lipoprotein uptake, regulation of bile acid synthesis, and how lipids are transported both within the cell as well as out of the cell in the form of lipoproteins and biliary bile salt/cholesterol/phospholipid micelles. Questions that were to be addressed include:

1) *Chylomicron remnant uptake.* Since chylomicron remnants do not accumulate in LDL receptor-negative patients, it seems likely that a receptor other than the apoB/E (LDL) receptor is involved in the uptake of chylomicron remnants. What are the processes responsible for chylomicron remnant uptake by the liver?

2) *HDL uptake.* Most studies show that incubation of cells (including hepatocytes) with apoE-free HDL causes a decrease in cellular cholesterol concentrations. How is cholesterol, initially associated with apoE-free HDL, delivered to the liver to complete the "reverse-cholesterol transport" cycle?

3) *Bile acid synthesis.* Several studies show that bile acids do not inhibit bile acid synthesis via a direct interaction with the hepatocyte. However, it is clear that bile acid synthesis varies in parallel to cholesterol synthesis and lipoprotein uptake. What factors determine whether cellular cholesterol is converted to bile acids, is esterified, is secreted as cholesterol in bile, or is assembled into lipoproteins?

4) *VLDL assembly.* With the major accomplishment of delineating the entire coding sequence of apoB, many of its unusual structural features have become known. How do these unusual features (i.e., large size, hydrophobicity, concentration of disulfide bonds on the N-terminus, and variable molecular weight forms) relate to the processes through which VLDL is assembled and secreted from the hepatocyte?

Daniel Steinberg summarized new data suggesting that oxidatively modified LDL may play an important role in atherogenesis. Incubation of LDL in the presence of Cu^{2+} or with endothelial cells results in the production of a modified LDL that is recognized by the acetyl-LDL receptor found on macrophages. The modification process includes the oxidation of lipids (primarily phospholipid) and the alteration of apoB. Oxidized LDL has the ability to compete with acetylated LDL for binding to macrophages. The uptake by the macrophage acetyl-LDL receptor leads to the accumulation of cholesteryl ester within the cell. In addition, it was shown that this uptake activates ACAT. Data showing that probucol inhibits the oxidation of LDL was presented. Dr. Steinberg suggested that preventing the oxidation of LDL may reduce atherogenesis.

Robert Mahley described the processes which may be involved in the uptake of chylomicron remnants by the liver. The postulated receptor for chylomicron remnants has been referred to as the apoE receptor. Binding assays performed

Abbreviations: VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; ACAT, acyl CoA: cholesterl acyltransferase; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl CoA reductase; HMG-CoA synthase, 3-hydroxy-3-methylglutaryl CoA synthase.

with canine and human liver membranes have detected the presence of an apoE-binding protein(s) that bind(s) apoE-containing lipoproteins (chylomicron remnants and apoE-HDL_c) with very high affinity. ApoE-free LDL did not bind to this protein. The protein was isolated and initially thought to be a single protein having a molecular weight of approximately 56,000. Monoclonal antibody studies performed in collaboration with Ulrike Beisiegel suggest that the apoE binding was due to more than one protein. At least three proteins displaying high affinity for binding apoE have now been identified: *1*) proteins having molecular weights between 52,000 to 55,000 have been tentatively identified as the α and β subunits of F^1-ATPase; *2*) a 59,000 molecular weight protein that is not an ATPase; *3*) a cloned protein with a molecular weight greater than 50,000. This latter protein is a structurally interesting protein that possesses a region enriched in amino acids resembling the region of the binding domain of the apoB,E (LDL) receptor. Therefore, there are several apoE-binding proteins in the liver. However, at the present time it is not known which, if any, of these proteins is responsible for the physiological uptake of chylomicron remnants.

Scott Grundy described the physiologic implication of the hepatic lipoprotein uptake. Studies in Dr. Grundy's laboratory have shown that there are two major mechanisms for elevated plasma concentrations of LDL-cholesterol. These are defective clearance of LDL and excessive input of LDL. Both processes have been found to be complex and multifactorial. Clearance of LDL appears to be a function of both the activity of LDL receptors and the properties of LDL itself. Patients with heterozygous familial hypercholesterolemia have an abnormality in the gene encoding LDL receptors which results in expression of only half the normal number of receptors. However, the majority of patients with primary hypercholesterolemia of the nonfamilial type appear to have a delayed clearance of LDL. These patients probably have a metabolic defect leading to a reduced synthesis of LDL receptors, i.e., a regulatory defect rather than a defect in the gene encoding the LDL receptor. A minority of patients with hypercholesterolemia have been found to have LDL particles having a reduced affinity for LDL receptors. In one family, this condition has been demonstrated to be a genetic defect, and it appears to be inherited as a monogenetic dominant disorder. Finally, another group of patients with hypercholesterolemia have an over-production of LDL. One cause of an excessive production of LDL is a reduced activity of LDL receptors, which leads to reduced uptake the VLDL remnants, the precursor of LDL. Consequently, more VLDL is converted into LDL. Patients of this type can be readily identified beause they have a reduced fractional catabolic rate for LDL. On the other hand, patients with an overproduction of LDL have normal fractional catabolic rates for LDL, and they seemingly have an excessive amount of hepatic secretion of the LDL precursor.

SESSION I. REGULATION OF HEPATIC CHOLESTEROL SYNTHESIS

Peter Edwards, Session Chairman

Kenneth Luskey described his studies on the regulation of HMG-CoA reductase. Promoters for HMG-CoA reductase have been constructed in which the nucleotides of different adjacent sequences have been scrambled. The ability of these modified promoters either to act as functional promoters (when coupled to the chloramphenicol acetyl transferase gene) or to bind *trans*-acting factors, as assessed by footprint analyses, have identified specific regions of the promoter that may play a role in transcriptional control of the gene.

The reductase protein appears to span the membrane of the endoplasmic reticulum several times. When the enzyme is expressed at very high levels, the cell accumulates crystalloid endoplasmic reticulum. Cellular cholesterol is known to normally enhance the degradation of HMG-CoA reductase. Luskey used genetic manipulations to delete two of the proposed membrane-spanning segments. This modified reductase protein altered the morphology of the endoplasmic reticulum and, in addition, the rate of enzyme degradation was no longer enhanced by cellular sterols. These experiments show the importance of the protein structure in determining the expression of this key enzyme cholesterol synthesis.

Peter Edwards discussed studies showing that, in rat liver, two other enzymes were coordinately regulated with HMG-CoA reductase. HMG-CoA synthase was purified, and antibodies and cDNA were then obtained. In addition, plus/minus screening of a rat liver cDNA library led to the isolation of a cDNA (CR39) that hybridized to an mRNA that was coordinately regulated with the mRNAs for HMG-CoA reductase and HMG-CoA synthase under various dietary conditions. CR39 was shown to encode a 39,000 dalton polypeptide. Sequence analyses of the full-length cDNA indicated that it contained an open reading frame encoding a protein of 39,615 daltons. An internal 30 amino acid sequence of CR39 showed significant homology (17 out of 30 residues) with the active site peptide of chicken prenyl transferase isolated by Dr. Hans Rilling and colleagues. Preliminary results indicated that a fusion protein of TrpE-rat CR 39 was recognized by an antibody raised against pure chicken prenyl transferase. It was proposed that HMG-CoA, HMG-CoA reductase, and CR 39 (prenyl transferase) are coordinately regulated.

Immunohistochemistry was used to demonstrate that, in the livers of normal fed rats, HMG-CoA reductase and HMG-CoA synthase were expressed in high levels in cells located periportally. These data further support the proposed coordinate regulation of these enzymes and raise the intriguing question of whether these specific periportal cells have a unique function in bile acid biosynthesis, and/or lipoprotein metabolism.

Michael Sinensky used inhibitors and mutants of cholesterol synthesis to demonstrate that regulation of HMG-CoA reductase activity required both sterols and mevalonate-derived non-sterols. It was proposed that the non-sterol regulator must be synthesized in order for sterols to exert their full regulatory effect on this enzyme. At present, the natures of the regulator sterol and the non-sterol are unknown. A number of laboratories are attempting to isolate and characterize these regulators. Using slot blots and Northern blots it was shown that the non-sterols regulate reductase activity by translational control. This translational control by non-sterols appears to be synergistic with the control exerted by sterols. In addition, data were presented showing that radioactive mevalonate was rapidly incorporated into a number of proteins. The linkage between the protein and the isoprenylated units may involve a cysteine residue. It was hypothesized that specific isoprenylated proteins may have a role in the regulation of HMG-CoA reductase.

SESSION II: LIPID TRANSPORT

Donald Small: Session Chairman

Robert Simoni presented data describing the intracellular transport of lipids. Chinese hamster ovary (CHO) fibroblast cells were given [^{14}C]acetate over a long period of time to label the cholesterol pool to constant specific activity. The cells were then pulsed for 4 min with [^{3}H]acetate and then cells were isolated at various times after a chase with unlabeled acetate. Cells were fractionated on a sucrose gradient into a number of fractions. Most dense to least dense were: endoplasmic reticulum (ER), plasma membrane, Golgi, and a lipid-rich vesicle fraction (LRV). At 37°C, the ^{3}H/^{14}C ratio reached a roughly constant ratio in 20–30 min, indicating equilibration of newly synthesized cholesterol in the cell. However, when cells were incubated at 15°C during the chase, the transport of free cholesterol slowed and newly synthesized cholesterol accumulated in both the ER fraction and the LRV fraction. Using a 15°C block, vesicular stomatitis virus G protein (VSV-G) was also found in the LRF fraction after 30 min. The oligosaccharide structure of G-protein indicates that these vesicles represent a pre-Golgi compartment. Digitonin was used to shift the density of vesicles containing *high* levels of free cholesterol. The results were consistent with two pools of vesicles in the LRV fraction: one pool contained low amounts of cholesterol, was not shifted by digitonin treatment, and contained the newly synthesized G-protein and newly synthesized cholesterol; a second pool of vesicles was shifted by digitonin treatment, was enriched in cholesterol, and probably represents plasma membrane contamination. It was concluded that since cholesterol synthesis occurs in the ER, cholesterol is then transported to the LRV fraction. Presumably the LRV fraction forms from budding of the ER. Cholesterol then moves to the Golgi and finally to the plasma membrane. In general, newly synthesized cholesterol takes 15–90 min to reach the plasma membrane from its site of synthesis.

Yvonne Lange described research elucidating the mechanisms that determine the distribution of cholesterol within cells. Cholesterol oxidase was used as a probe to distinguish intracellular cholesterol from cholesterol-rich plasma membranes in various cultured cells. Typically, more than 90% of cellular cholesterol was attributable to the plasma membrane. It was shown that newly synthesized cholesterol moves to the plasma membrane in a vectoral fashion that is under metabolic control. Evidence for the existence of a segregated compartment of newly synthesized cholesterol within the cytoplasm was obtained in a study of steroidogenic cells. Subcellular fractionation of biosynthetically labeled fibroblasts showed that: *1*) intracellular cholesterol in transit to the plasma membrane is associated with cholesterol-rich membrane; *2*) newly synthesized lanosterol and cholesterol are not in the same membranes as HMG-CoA reductase; and *3*) when cells are labeled with [^{14}C]acetate at 10°C, newly synthesized lanosterol accumulates in a buoyant membrane fraction. It was hypothesized that the pathway of steroid biosynthesis is topographically heterogeneous with its inception in the endoplasmic reticulum and its completion coupled with the delivery of cholesterol at the cell surface.

Louis Smith described his studies on the transport mechanisms determined by computer-enhanced analysis of fluorescent analogs. The kinetics of cellular uptake of a fluorescent fatty acid, 12 (3-pyrenyl) dodecanoic-acid (PC 12:0), was determined in HepG2 cells and in 3T3 L-1 cells by digital imaging fluorescent microscopy. The fluorescent fatty acid was incorporated into all classes of cellular lipid, with a time course of distribution comparable to that of oleic acid in HepG2 cells. Living cells were incubated on the microscope stage with PC 12:0/alkane complex (2:1 mole ratio) and with 1-palmitoyl-2-oleic phosphatidylcholine containing 2 mol % PC 12:0. Fluorescent images were acquired at 20-min intervals for 203 hr. Mapping the rate constant revealed as much as a 300-fold range of rate constants within a single cell, with wide variations in the spatial locations of the highest rate constants in each cell. Optical sectioning of the cells indicated that the location of the highest rate constants for uptake was distinct from lysosomes visualized by acridine orange, from lipid droplets visualized by Nile red, and in close proximity to the nucleus identified by Hoescht staining. Fixation of cells with 4% (v/v) formaldehyde before response to fatty acid uptake produced a surface distribution of PC 12:0. The overall rate of cellular uptake was reduced 3- to 4-fold. Little, if any, spatial variation in the rate constants for uptake was observed.

Alan Attie described his experiments on the intracellular transport and membrane flip/flop of cholesterol. Fresh erythrocytes were labeled for 5 min with [^{3}H]cholesterol at 4°C to selectively label the outer leaflet of the membrane.

Radiolabeled erythrocytes were suspended in low ionic strength buffer containing 1 mM magnesium, and were then incubated at 37°C to allow an equilibration of [^{3}H]cholesterol across the membranes. At various times, aliquots of the cells were removed and treated briefly for 20 sec with cholesterol oxidase to convert a small portion of the outer leaflet cholesterol to cholestenone. The rate of decrease in the specific radioactivity of cholestenone represented the rate of transbilayer movement of cholesterol. The rate of transbilayer movement of cholesterol had a $t_{1/2}$ of 49 min at 37°C. Additionally, continuous incubation of radiolabeled erythrocytes in the presence of cholesterol oxidase at 37°C caused an acceleration of the rate of transbilayer movement of cholesterol ($t_{1/2}$ = 6 min). These data also suggest that cholesterol has an asymmetric distribution in erythrocyte membranes with the majority of the cholesterol being in the inner leaflet.

SESSION III: INTRAHEPATIC ASSEMBLY OF LIPOPROTEINS

Godfrey Getz, Session Chairman

Lawrence Chan described exciting new data on the structural and functional domains of apoB. C-Y. Yang at Baylor has mapped the trypsin-accessible sites of apoB-100 by digestion of LDL particles with the enzyme. The released peptides (trypsin-accessible) were fractionated on Sephadex G-50, followed by HPLC. The undigested portion of apoB-100 was delipidated and redigested with trypsin. The tryptic peptides from the digestion (labeled trypsin-accessible and -inaccessible peptides) were purified on two HPLC columns. All the trypsin-accessible and -inaccessible peptides recovered from the HPLC columns were sequenced. To date, about 85% of apoB-100 has been sequenced by this approach. The distribution of these peptides on LDL is nonrandom and they can be grouped into five hypothetical domains, whose characterization will be useful to the understanding of apoB-100 conformation on LDL. The structure of human apoB-48 was deduced by four complementary approaches: DNA-excess hybridization of small intestinal mRNA using apoB-100 cDNA probes; direct sequencing of apoB-48 trypsin peptides; sequencing of cloned intestinal apoB cDNAs; and direct sequencing of intestinal mRNA. ApoB-48 was shown to be a product of an intestinal mRNA that has an in-frame UAA stop codon resulting from a C to U change in the codon on CAA encoding Gln-2153 in apoB-100 mRNA. The COOH-terminus Ile-2152 of apoB-48 purified from chylous ascites fluid was apparently cleaved from the initial translation product, leaving Met-2151 as the new COOH-terminus. DNA-excess hybridization and sequencing data indicated that approximately 85% of the intestinal mRNAs terminate within approximately 110 bases downstream from the stop codon. The other approximately 15% of the mRNAs had lengths similar to hepatic apoB-100 mRNA, but they also had the same in-frame stop codon. The organ-specific introduction of a stop codon into an mRNA appears unprecedented, and might have implications for the utilization of cryptic polyadenylation signals and RNA processing.

Roger Davis presented novel data on the intrahepatic processing of rat apoB. Data showed that apoB binds to actin filaments especially in the endoplasmic reticulum, while most of the apoB in the Golgi fractions is not actin-bound. A monoclonal antibody (HB 41) was prepared and shown to react only with apoB-100 and not with plasma apoB-48. In the rough endoplasmic reticulum, this monoclonal antibody reacted with peptides having a size similar to or smaller than that of apoB-100. In the Golgi fraction, on the other hand, this monoclonal antibody reacted almost exclusively with apoB-100. Davis proposes that apoB-100 in the rough endoplasmic reticulum has an NH_2-terminal domain within the lumen of the endoplasmic reticulum, while the COOH-terminal domain extends from the endoplasmic reticulum into the cytoplasm and this may be subjected to proteolysis, leaving apoB-48 inside the endoplasmic reticulum and the remainder of the apoB (apoB-52) unable to gain entry into the secretory pathway because of its lack of a signal sequence.

Dennis Vance discussed the relationship between phosphatidylcholine biosynthesis, and lipoprotein secretion in cultured hepatocytes. Phosphatidylserine appears to be a better precursor for lipoprotein phosphatidylcholine than is phosphatidylethanolamine. Phosphatidylcholine and phosphatidylethanolamine biosynthesis via the CDP-choline and CDP-ethanolamine pathways were shown to be localized in the Golgi fractions as well as in the endoplasmic reticulum. On the other hand, phosphatidylserine decarboxylase catalyzing the formation of phosphatidylcholine and phosphatidylethanolamine is mainly localized in the mitochondria and is not found in the Golgi apparatus. It remains an enigma as to how phosphatidylethanolamine and phosphatidylcholine derived from this decarboxylation are preferentially associated with secreted lipoproteins. Evidence was also presented to show that phosphatidylcholine biosynthesis was required for the secretion of VLDL but not HDL from cultured rat hepatocytes. There was a greater than 50% reduction in the secretion of lipids and apolipoproteins associated with VLDL in hepatocytes derived from choline-deficient rats. The reduced secretion was fully reversed by the addition of choline or methionine to the medium. The turnover of apolipoproteins in the hepatocyte was unaffected by choline and methionine. It was postulated that phosphatidylcholine synthesis was required for the secretion of apoB into the lumen of the endoplasmic reticulum.

Godfrey Getz discussed work done in collaboration with Drs. T. Mazzone and J. Schreiber on the regulation of apoE biosynthesis in extrahepatic tissues (cholesterol-loaded mouse peritoneal macrophage, and hormone-stimulated rat ovarian granulosa cells). Mouse peritoneal macrophages

loaded with cholesterol from acetyl LDL showed an increase in apoE biosynthesis and in RNA levels. Evidence was presented to show that changes in cellular cholesterol concentration induced by incubation of cells with HDL and an ACAT inhibitor correlated with changes in apoE biosynthesis. ApoE biosynthesis in isolated ovarian granulosa cells was stimulated by follicle-stimulating hormone and other agents that induced an increase in cellular cAMP concentration. While the abundance of apoE mRNA increased, this did not appear to account for all the increased apoE secretion. Mevinolin inhibited the cAMP stimulation of apoE biosynthesis. It was suggested that a mevalonic acid-derived product, perhaps cholesterol, mediates or is required permissively for this apoE synthesis. HDL, provided as an exogenous source of additional cholesterol to cAMP-stimulated cells, markedly increased apoE secretion. However, there was little change in apoE mRNA abundance, suggesting that the effect of HDL on apoE synthesis was at a posttranscriptional step. Stimulation of an ovarian granulosa cell with phorbol esters also resulted in an increment in apoE biosynthesis and RNA levels. The time course for this response and modest changes in steroidogenesis indicate that the effects of phorbol ester are not mediated by cAMP, though the latter may potentiate effects of the ester. It is postulated that cAMP and phorbol ester promote the phosphorylation and activation of putative transcription factors to increase apoE gene transcription in ovarian granulosa cells.

SESSION IV: REGULATION OF ACAT

Alan Fogelman, Session Chairman

Ira Tabas reported that J774 macrophages accumulate cholesteryl ester in the presence of native LDL, whereas mouse peritoneal macrophages accumulate cholesteryl ester in the presence of modified LDL (e.g., acetyl-LDL) or β-VLDL. To further understand this phenomenon with respect to intracellular cholesterol metabolism, J774 macrophages and mouse peritoneal macrophages were incubated under conditions in which LDL uptake and degradation and cholesteryl ester hydrolysis were identical in the two cell types. Despite this equal delivery of LDL-derived cholesterol to the different cell types, ACAT activity was 10-fold higher in the J774 macrophage. This was not due to the lack of ACAT in the mouse peritoneal macrophages, since ACAT activity was very high when the cells were exposed to acetyl-LDL. However, native LDL was able to markedly stimulate ACAT in mouse peritoneal macrophages (and cause cholesteryl ester accumulation) in the presence of cycloheximide and other inhibitors of protein synthesis. The cycloheximide effect occurred rapidly (4 hr) and was also rapidly reversed (approximately 4 hr) after cycloheximide removal. LDL stimulation of ACAT in J774 cells was not stimulated by cycloheximide. From these and other data it was hypothesized that: *a*) the lack of ACAT stimulation by LDL in mouse peritoneal macrophages is neither due to a lack of LDL receptor activity nor to a lack of ACAT, but rather a failure of LDL-cholesterol to be delivered to ACAT; *b*) acetyl-LDL and LDL may be delivered to different sites in the mouse peritoneal macrophages such that only cholesterol of acetyl-LDL is efficiently delivered to ACAT; *c*) the inefficient delivery of LDL-cholesterol to ACAT in mouse peritoneal macrophages may be due to the presence of a short-lived protein(s) that may, for instance, function to transport LDL-cholesterol to a non-ACAT site (e.g., the plasma membrane); and *d*) the marked ability of LDL to stimulate ACAT in J774 macrophages may be due to the lack of this putative short-lived protein(s) in these cells.

Peter Pentchev spoke about type C Niemann-Pick disease which is an autosomal recessive neuro-visceral lipid storage disorder. Cultured fibroblasts from affected individuals accumulate excessive amounts of unesterified cholesterol when incubated with LDL. He reported that delivery of cholesterol from lysosomes to other subcellular sites was deficient in the Niemann-Pick C cells. The uptake of unesterified cholesterol was also associated with deficient regulatory responses on the part of the affected cells. Studies of these mutant cells may allow new insights into the distribution of cholesterol to ACAT.

Brian Van Lenten described a subcellular fractionation of human monocyte-macrophages that allowed him to follow lipoproteins through the endocytotic-lysosomal pathway. He also reported that increasing the phospholipid content of LDL resulted in increased ACAT activity and he hypothesized that this may have resulted from an increased transport of cholesterol from the lysosomal compartment to ACAT-containing microsomes.

T.Y. Chang reported on a reconstituted in vitro ACAT assay for Chinese hamster ovary (CHO) cells. This method allows ACAT activity to be measured independently of the enzyme's original lipid environment. Extracts prepared from cells incubated under different conditions had ACAT activities that varied 20-fold. The differences in the activity were abolished in the reconstituted assay, implying that modulating the amount of ACAT enzyme is not an important mechanism for regulating ACAT activity by sterols in CHO cells. Further studies using mutant cells defective in regulation of cholesterol metabolism supported this view. Chang also presented evidence suggesting the existence of a short-lived cellular factor(s) which serves as an endogenous ACAT inhibitor. This factor(s) may inhibit the enzyme directly or may work by preventing cholesterol delivery to the enzyme. He described a method for isolating CHO cell mutants defective in cholesteryl ester synthesis. Biochemical analysis revealed that they were defective in ACAT activity. Using the ACAT-deficient mutant as the recipient, he reported on the isolation of a primary transfectant that regained ACAT activity following DNA-mediated gene transfer using high molecular weight DNA from cultured human fibroblasts.

SESSION V: MECHANISMS AND REGULATION OF CHOLESTEROL SECRETION

Fred Kern, Jr., Session Chairman

Thomas Holzbach talked about the vesicular transport of cholesterol in bile. The concept has recently emerged that phospholipid- and cholesterol-containing vesicles (in addition to bile salt-lecithin micelles) represent a significant supplementary particle responsible for solubilizing and transporting cholesterol in bile. Agreement on this point has now been reached in reports from at least five groups using a variety of methods for isolation and structural assessment. It is clear that the "newly discovered" vesicles are merely an updated version of structures observed previously and at that time referred to as liquid crystals or mesophase. Recognition of vesicles and their role in cholesterol secretion and transport helps explain the metastable supersaturation possible in these systems without cholesterol crystal formation. Studies have demonstrated that there are several factors that can affect vesicle formation and composition. Dilution is of key importance in favoring vesicle formation. This explains the dominance of vesicle over micelles for cholesterol transport in vivo in dilute biological systems such as human and rat bile. With concentration of lipid in human gallbladder, vesicles are converted to micelles and the latter then become the dominant cholesterol transporting particle. Other factors increase the cholesterol content of vesicles, making them more unstable and favoring cholesterol crystal formation by nucleation. Vesicles in dilute systems are cholesterol-poor and accordingly extremely stable. Increased cholesterol saturation and increased bile salt-lecithin molar ratios are also compositional factors in bile that produce unstable nucleation-prone, cholesterol-rich vesicles. An unproven but reasonable view is that nucleation-promoting and nucleation-inhibiting (probably protein) factors in bile may mediate their activities through interaction with vesicles. A final new concept derived from recent recognition of vesicles in bile is that their aggregation is closely linked to the cholesterol crystal nucleation process which presages human cholesterol gallstone formation.

Christian Drevon discussed the effect of eicosapentaenoic acid on synthesis and secretion of cholesterol and cholesteryl esters by cultured rat hepatocytes. In the presence of eicosapentaenoic acid, cellular cholesterol esterification was reduced by 50–75% compared to experiments with oleic acid as measured by radioactive precursors and mass. The decreased synthesis of cholesteryl esters was observed within 1 hr and lasted for at least 20 hr. Eicosapentaenoic acid reduced cholesterol esterification in the concentration range of 0.2 to 1.0 mM fatty acid and reduced the stimulatory effect of oleic acid on cholesterol esterification. Secretion of cholesterol into the medium was reduced by 50–60% in the presence of eicosapentaenoic acid. Cellular and secreted free cholesterol were unaffected by eicosapentaenoic acid as compared to oleic acid. Cholesterol esterification and release of cholesteryl ester were markedly increased by 25-hydroxycholesterol in the presence of eicosapentaenoic acid as well as oleic acid. Experiments with liver microsomes revealed that radioactive eicosapentaenoic acid and eicosapentaenoyl-CoA were poorer substrates (7–30%) for cholesterol esterification than oleic acid and oleyl-CoA. Reduced formation of cholesteryl ester was also observed when eicosapentaenoyl-CoA was given together with oleoyl-CoA. Drevon concluded that eicosapentaenoic acid reduced cellular cholesterol esterification by causing decreased activity of ACAT. The lower cholesterol esterification caused by eicosapentaenoic acid leads to reduced secretion of VLDL cholesteryl ester.

Flavio Nervi explored the possibility that biliary cholesterol output may be dependent on intrahepatic processes that determine the flux of free cholesterol from the metabolic pool through hepatic excretory pathways: VLDL and HDL secretion and bile acid excretion. Nervi and coworkers performed in vivo experiments in male rats and in the isolated perfused rat liver. The three excretory pathways were manipulated with different diets known to influence either in vivo output (fructose), bile acid synthesis (colestipol), or biliary cholesterol output (diosgenin). It was found that *1*) fructose decreased biliary cholesterol output, increased VLDL cholesterol output, and did not affect bile acid synthesis; *2*) colestipol increasd bile acid synthesis, but biliary cholesterol and VLDL and HDL cholesterol outputs remained unchanged; *3*) diosgenin markedly increased biliary cholesterol output, inhibited the corporation of [^{14}C]oleate into hepatic cholesteryl esters, and decreased VLDL free and esterified cholesterol. Bile acid synthesis remained unchanged. These studies suggest that biliary cholesterol and VLDL cholesterol originated from a common intrahepatic pool which was separate from the metabolic pool that supplies cholesterol to the bile acid synthetic pathway. In addition, these studies reinforce the concept that the rate of biliary cholesterol is markedly influenced by the availability of free cholesterol in the metabolic pool of the hepatocyte. The biliary and VLDL cholesterol excretory pathways are reciprocally interrelated.

Fred Kern described an investigation into the mechanism through which contraceptive steriods increase biliary cholesterol secretion and bile lithogenicity. Healthy premenopausal women were studied while on and off low doses of estrogen steroids (35 μg). During the use of contraceptive steroids, cholesterol saturation of gallbladder bile and the amount of cholesterol secreted per mole of bile acid increased significantly. Cholesterol absorption, cholesterol synthesis, chylomicron remnant clearance, and the concentration of plasma and lipoprotein lipid were not altered by contraceptive steriods. Despite this apparent lack of effect on regulatory mechanisms, statistically significant correlations were present during steriod use. LDL cholesterol increased as dietary cholesterol increased.

Cholesterol synthesis correlated with VLDL cholesterol concentration, biliary cholesterol secretion, and mole percent cholesterol in bile. Chylomicron remnant clearance also correlated with cholesterol secretion. As either remnant uptake or synthesis increased, the effect of the other source of hepatic cholesterol biliary cholesterol secretion diminished. These relationships were not observed in the same subjects when they were not taking the hormones. The findings suggest that both newly synthesized and dietary cholesterol contributed to cholesterol secreted in bile, consistent with the hypothesis that cholesterol for secretion into bile and VLDL is derived from a common metabolic pool of free cholesterol. It was proposed that contraceptive steroids exert their effect on biliary cholesterol by increasing the cholesterol entering the pool and/or by inhibiting hepatic ACAT activity, a known effect of progesterone, so that an increase in free cholesterol entering the pool leads to an increase in output.

SESSION VI: CONCLUSIONS AND FUTURE DIRECTIONS

Daniel Steinberg, Session Chairman

Presentations during this conference demonstrated significant progress with respect to our understanding of regulation of hepatic cholesterol synthesis, biosynthesis, and catabolism of lipoproteins, and bile acid synthesis and secretion. However, a number of important questions remain unresolved. Those that the conferees considered particularly worthy of intensive investigation included the following.

1) Receptor-mediated uptakes of lipoproteins. While the apoB/E receptor described by Brown and Goldstein has been elegantly characterized, much remains to be learned about the acetyl-LDL receptor, the β-VLDL receptor, and chylomicron remnant (apoE only) receptor. Dr. Mahley reported that his group has now shown that one apoE-binding protein that they have extensively categorized is actually a mitochondrial membrane ATPase. Clearly, additional intensive studies are warranted to establish the mechanisms of chylomicron remnant binding and uptake into the liver.

New data were reported by Steinberg suggesting that uptake of oxidatively modified LDL by way of the acetyl-LDL receptor may play an important role in atherogenesis. However, the acetyl-LDL receptor has yet to be fully characterized and cloned.

Finally, despite a good deal of research on the binding of HDL and some preliminary reports of specific ligand-binding proteins, there is no firm evidence for a specific HDL receptor linked to a specific function. This area is of particular importance because of the proposed role of HDL in reverse cholesterol transport.

2) Receptor-independent lipoprotein uptake. Uptake of LDL in normal individuals occurs predominantly (60–70%) by way of the apoB/E receptor. Very little is known about the other 30–40% and still less about the 100% taken up by LDL receptor-independent mechanisms in homozygous familial hypocholesterolemics. The extent to which receptor-independent uptake contributes to plasma clearance of lipoproteins remains unclear.

3) Reverse cholesterol transport. While it is undeniably the case that cholesterol *must* be returned in some fashion from peripheral tissue to the liver for catabolism and excretion, the rate at which this occurs and the precise mechanisms are yet to be delineated. It seems highly likely that LCAT is involved and that the transport occurs on HDL. What we do not know is the extent to which cholesterol moves from HDL to other lipoprotein fractions prior to hepatic uptake and the extent to which direct hepatic uptake of HDL is involved. How much of the latter represents uptake of the intact HDL and how much uptake occurs by the newly described selective uptake of cholesteryl esters? Since aberrations in the reverse cholesterol transport could play a critical role in susceptibility to atherosclerosis, as in the case of patients with complete absence of apoA-I, it would be important to know more about the mechanism involved.

4) Lipoprotein biosynthesis. Available data strongly suggest that over-production of lipoproteins accounts for hypercholesterolemia in many clinical syndromes, probably including familial combined hyperlipidemia. Despite a good deal of research effort, we still cannot describe in detail the intimate mechanisms by which lipoproteins are put together and are secreted by the liver. Dr. Davis presented an intriguing new hypothesis regarding the transport of apoB into the lumen of the endoplasmic reticulum. More studies should be carried out to test this and other hypotheses concerning the biogenesis of VLDL and its transport to the surface of the hepatocyte. New approaches, including immunoelectron microscopy and somatic cell genetic approaches, might be employed.

5) Intracellular channelling of lipids. In a sense this problem represents a subset of problems relating to cytoskeletal structure and the movement of cellular components from point to point in the cell. In addition to its intrinsic interest as a problem in cell biology, there may be crucially important physiologic aspects. For example, the ability of cells to control their cholesterol content may be limited by the rate at which stored cholesteryl ester can be made available at the cell surface for reverse cholesterol transport. Further information is needed both about the mechanisms involved and about the rate of the transfer processes.

Clinical aspects of hypercholesterolemia. Now that the nature of the LDL receptor has been firmly established and broad outlines of LDL receptor defects have been worked out, we can anticipate the elucidation of more subtle abnormalities in receptor structure and function. Concurrently, we can expect that more subtle aberrations in lipoprotein structure will be described that are relevant to hypocholesterolemia and atherosclerosis. Dr. Grundy described a clear

example of apoB polymorphism that profoundly influences LDL removal. Dr. Young described another obvious abnormality of apoB structure in a kindred with hypobetalipoproteinemia. Through the use of molecular biology approaches and through careful clinical studies utilizing kindred with abnormal lipoprotein metabolism, we can expect to uncover a complex array of abnormalities relating to lipoprotein structure and to the structure/function of receptors.

Final note. It was concluded that increased efforts should be made towards gaining an understanding of the role of the liver in atherosclerosis. In response to this conclusion, the organizing committee decided to hold a yearly conference on this topic in Aspen, Colorado. Peter Edwards at the University of California, Los Angeles and Scott Grundy at the University of Texas Health Sciences Center in Dallas, Texas were chosen as Chairmen for the next conference (August 18–21, 1988) which is entitled "Hepatic Cholesterol and Lipoprotein Conference." Persons interested in participation in this conference should contact Dr. Edwards or Dr. Grundy.

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