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1992 Aspen Bile Acid/Cholesterol/Lipoprotein Conference. Report of a Conference

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The 1992 Aspen Bile Acid/Cholesterol/Lipoprotein Conference "Regulation of hepatic cholesterol Transport and Metabolism" was held at the Given Institute of the University of Colorado School of Medicine in Aspen, Colorado on August 15-18, 1992.

Dr. John Chiang opened the meeting with a discussion of the key bile salt synthetic enzyme cholesterol 7α-hydroxylase. Using an HPLC-based assay method, a specific antibody against purified 7α-hydroxylase, and cDNA hybridization probes, he demonstrated that the induction of 7α-hydroxylase activity in rat liver by cholestyramine or bile fistula is due to a parallel increase in enzyme mass, mRNA, and the transcription rate of 7α-hydroxylase gene. Infusion of hydrophobic bile salts, on the other hand, reduced the rate of gene transcription, thus the activity, protein, and mRNA levels. He demonstrated that cholesterol, thyroid hormone, or glucocorticoid stimulated cholesterol 7α-hydroxylase enzyme activity, protein, and mRNA levels in rat liver by increasing the rate of cholesterol 7α-hydroxylase gene transcription. He concluded that the major mechanism regulating 7α-hydroxylase activity in the liver is at the gene transcriptional level. The heterologous expression of catalytically active cholesterol 7α-hydroxylase in *E. coli* has been achieved. A truncated enzyme lacking the N-terminal membrane-binding domain was expressed at high level and was isolated to study enzyme kinetics and the effects of oxysterols on cholesterol binding and regulation of 7α-hydroxylase. The truncated form had the same K_m for cholesterol but lower V_{max} than the wild-type 7α-hydroxylase. Therefore, the membrane-binding N-terminal sequence is not required for catalysis. 7-Ketocholesterol or 7α-hydroxycholesterol competitively inhibited cholesterol binding to the enzyme, whereas 7β-hydroxycholesterol had no effect. Infusion of 7-ketocholesterol to the rat increased 7α-hydroxylase activity, protein, and mRNA levels by two- to threefold in the rat liver. He proposed that oxysterol competitively inhibited 7α-hydroxylase activity and thus reduced bile acid synthesis and feedback inhibition of 7α-hydroxylase. As a result, the expression of 7α-hydroxylase is induced to compensate for the decrease in bile acid synthesis. This is similar to the induction of HMG-CoA reductase activity and mRNA by mevinolin. To study the gene regulation by physiological regulators, genomic clones of the rat, hamster, and human cholesterol 7α-hydroxylase genes have been isolated and nucleotide sequences of these genes have been determined. The 5'-flanking region of the rat cholesterol 7α-hydroxylase gene contains many hormone-responsive elements, liver-specific or -enriched elements, ubiquitous transcription factor-binding elements, and the "CA" repetitive motifs. The latter motifs have potential to form a Z-DNA structure which has been demonstrated to repress gene transcription in other genes. The intron/exon organizations and structures are identical in these genes. However, a comparison of the 5'-flanking sequences of these genes revealed that promotor sequences about 250 bp upstream from the transcriptional start sites are highly conserved among these genes, but sequences became diverged further upstream. This indicates that the cholesterol 7α-hydroxylase gene may be regulated by somewhat different mechanisms in different animal models. He proposed that putative *cis*-acting regulatory elements may interact with inducible *trans*-acting protein factors and play roles in the regulation of cholesterol 7α-hydroxylase gene transcription by bile salts, cholesterol, and hormones. Possible mechanisms of gene activation were proposed, but the detailed molecular mechanisms of gene regulation are yet to be elucidated.

Dr. Roger Davis noted that the liver is the organ primarily responsible for the clearance of plasma lipoproteins and the catabolism of cholesterol to bile acids. Biliary excretion of cholesterol and bile acids provides the major pathway determining cholesterol homeostasis. The liver also exhibits resistance to down-regulation of the LDL receptor, thus maintaining an efficient uptake of LDL from plasma. He suggested that liver-specific expression of 7α-hydroxylase may provide a mechanism to maintain expression of the LDL receptor by metabolizing oxysterol repressors to bile acids and, thus, inactivating

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their ability to repress the expression of the LDL receptor. He examined this hypothesis in two different experimental models: L35 hepatoma cells that express 7α-hydroxylase in a regulatable manner and nonhepatic Chinese hamster ovary cells (CHO) transfected with an artificial gene that allows constitutive expression of 7α-hydroxylase. In L35 hepatoma cells in the absence of dexamethasone there is no expression of 7α-hydroxylase. However, adding dexamethasone caused a dramatic increase in the expression and mRNA for 7α-hydroxylase to levels observed in the livers of rats obtained during the highest point of the diurnal variation. The finding that dexamethasone also increases the expression of the LDL receptor mRNA supports the hypothesis that the expression of 7α-hydroxylase may indirectly regulate expression of the LDL receptor. To further examine this hypothesis, he transfected nonhepatic CHO cells with a plasmid encoding 7α-hydroxylase. Cells expressing 7α-hydroxylase were identified by both resistance to cytotoxic killing by 25-hydroxycholesterol and expression of 7α-hydroxylase mRNA and activity. 7α-Hydroxylase was functionally active in these cells as demonstrated by the formation of [^{14}C]7α-hydroxycholesterol from [^{14}C]acetate. In the presence of 5% serum, expression of LDL receptor mRNA was 20 times greater in two independent clones of CHO cells expressing 7α-hydroxylase than in nonexpression CHO cells despite a 40% increased content of cholesterol ester. Moreover, 7α-hydroxylase expressing CHO cells metabolized 25-hydroxycholesterol at a rate that was at least 4-fold greater than that exhibited by nonexpressing CHO cells. The combined data suggest that 7α-hydroxylase may preferentially use oxysterols as substrates, resulting in an induction of the LDL receptor. This may explain the relative resistance of hepatic LDL receptors to down-regulation.

Dr. Norman Javitt described experiments suggesting the presence of two 7α-hydroxylase enzymes. He plated HepG2 cells at high cell density and maintained in DMEM 4500 with 10% delipidated FBS. The cells synthesized both chenodeoxycholic acid and cholic acid. Addition of 5β-cholestane-3α,7α,12α-triol (THC) (25–100 μg) yields increasing amounts of cholic acid, thus indicating that the rates of both mitochondrial C27 bile acid formation and peroxisomal C24 bile acid synthesis are intact and in excess of endogenous production rates. The system is therefore rate-limited, probably attributable to cholesterol 7α-hydroxylase activity as occurs in vivo.

Addition of 27-hydroxycholesterol to the medium yields mostly the monohydroxy bile acid, 3β-hydroxy-5-cholenoic acid (54%) with less chenodeoxycholic acid (41%) and cholic acid (5%). The finding is in contrast to in vivo metabolism of 27-hydroxycholesterol which appears in bile mostly as chenodeoxycholic and cholic acids (>93%).

Using 2-hydroxypropyl-β-cyclodextrin as a vehicle it was possible to assay the activity of cholesterol 7α-hydroxylase and 27-hydroxycholesterol-7α-hydroxylase using cholesterol and 27-hydroxycholesterol, respectively, as substrates. No competitive inhibition was observed, indicating that two different cytochrome P-450 7α-hydroxylases are present in the microsomes. The activity of 27-hydroxycholesterol 7α-hydroxylase was much lower in HepG2 cells (14 pmol/min per mg protein) than in microsomal fractions of human liver (62 pmol/min per mg protein) thus accounting for the higher proportion of monohydroxy bile acid found in cell culture. Also, the ratio of the activity of 27-hydroxycholesterol-7α-hydroxylase/cholesterol 7α-hydroxylase was much higher in liver microsomes (19) than in HepG2 cells (0.1). Because the HepG2 cell line expresses all the enzyme activities needed for normal bile acid synthesis, it is an excellent model for studying determinants of its regulation.

Dr. Reno Vlahcevic noted that the presently available data suggest that hydrophobic bile salts repress cholesterol 7α-hydroxylase at the level of gene transcription; hydrophilic bile salts have no effect. Hydrophobic bile salts affect cholesterol 7α-hydroxylase directly rather than via primary suppression of HMG-CoA reductase, the rate-limiting enzyme in the cholesterol synthesis pathway. By contrast, cholesterol feeding (2% in diet) increases cholesterol 7α-hydroxylase activity, mass, mRNA levels, while inhibition of cholesterol synthesis decreases cholesterol 7α-hydroxylase mRNA levels and gene transcriptional activity, both in vivo and in vitro (primary cultured hepatocytes). Thyroxine and dexamethasone were found to increase cholesterol 7α-hydroxylase, mRNA, and transcription when added to the primary cultured hepatocytes. In contrast, glucagon and dibutyryl cyclic-AMP decrease in vitro transcription. The role of hormones was also tested in hypophysectomized animals; in these animals cholesterol 7α-hydroxylase activity, mRNA, and transcription were decreased by 50%. These in vitro and in vivo studies suggest that hormones also play a role in the regulation of cholesterol 7α-hydroxylase. Thus, it appears that cholesterol 7α-hydroxylase is regulated by a multitude of factors, some stimulatory, others inhibitory. While these data suggest that the major regulation of cholesterol 7α-hydroxylase occurs at the level of gene transcription, it is still not clear how these multiple factors interact under physiological circumstances.

Dr. Yvonne Lange discussed the intracellular distribution and movement of cholesterol. The following cholesterol compartments in cultured fibroblasts have been identified. *1)* About 90% of total unesterified cholesterol is in the plasma membrane. *2)* The major fraction of intracellular cholesterol, approximately 10% of the cell total, is in endocytic membranes. *3)* Nascent cholesterol, about 1% of the cell total, is found in discrete cholesterol-rich membranes not identifiable with any of the major organelles. *4)* Less than 0.1% of total cell unesterified cho-

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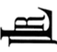

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lesterol is in the rough endoplasmic reticulum (RER). It appears that cholesterol circulates briskly between compartments within the cell. For example, newly synthesized cholesterol moves through the sterol-rich organelle to the plasma membrane with a half-time of about 20 min. In addition, the cholesterol derived from ingested lipoproteins, as well as that mobilized from ester stores, accumulates rapidly and exclusively in the plasma membrane. Reciprocally, plasma membrane cholesterol moves to the RER where it is esterified. Finally, late sterol intermediates, which are concentrated in the plasma membrane, return to the RER where they are converted to cholesterol; this nascent sterol then moves to the cell surface. A common mechanism may obtain in all these cases. How cholesterol is transported against its concentration gradient to the plasma membrane is our present concern.

Dr. Daniel Levy discussed the bile acid hepatic transport systems. Bile acids such as taurocholate are organic anions that play a critical role in numerous biological processes such as digestion, excretion of exogenous and endogenous substances, and in the regulation of cholesterol metabolism. Numerous studies have been concerned with the identification of the hepatocyte membrane protein(s) mediating the sodium-dependent uptake of these substrates across the basolateral membrane. The initial use of photoaffinity labeling procedures suggested the functional role of a 49 and/or 54 kDa protein in the transport process. The use of monoclonal antibodies further demonstrated the participation of the 49 kDa protein based on antibody modulation of the bile acid transport and immunoprecipitation procedures. This conclusion was further established using proteoliposome reconstitution procedures in conjunction with antibody reagents. Using *Xenopus* oocyte expression cloning, a 35 kDa hepatocyte protein has been shown to also mediate sodium-dependent bile acid uptake. The transport properties and substrate specificity of these two systems is under investigation. The 49 kDa bile acid transport system was also shown to be functionally expressed in vesicles derived from the smooth endoplasmic reticulum and to be mediated by a protein closely related to the 49 kDa protein located on the sinusoidal plasma membrane based on studies using antibody reagents and reconstitution procedures. These results suggest a role of intracellular vesicles in the transcellular movement of bile acids. Analysis of epitope accessibility suggested that the 49 kDa protein existed in the SER in two orientations, possibly resulting from alternative splicing. One of the isoforms is expressed on the hepatocyte surface while the other is retained in the ER.

The 49 kDa plasma membrane transport protein was purified to homogeneity by immunoprecipitation and HPLC. N-terminal amino acid sequence and amino acid composition revealed that this protein as well as the protein isolated from the ER was closely related to the enzyme microsomal epoxide hydrolase (mEH) exhibiting identical molecular weights, isoelectric points, enzymatic activity, and peptide profiles when fragmented with subtilisin. In order to directly establish the transport properties of this protein, the cDNA of mEH was prepared by PCR procedures and transfected into COS-7 cells. Expression of mEH was established by immunoprecipitation and enzymatic activity. Taurocholate uptake was evaluated and shown to be sodium-dependent and inhibited by a bile acid inhibitor (DIDS) and by bile acid derivatives. Mediated uptake in the transfected cells was shown to be at least 30-fold higher than in control cells. These results unequivocally establish that mEH is able to mediate bile acid transport across cell membranes. The structural motifs in isoforms of this protein leading to expression at multiple sites, multiple orientations in the ER, and different transport characteristic in the ER and PM are under investigation.

Dr. Andrew Stolz described his work on cytosolic bile acid binding proteins. Bile acids undergo a unique enterohepatic circulation characterized by efficient hepatic extraction with rapid excretion into the biliary system. Evidence from multiple lines of different experiments indicates that bile acids bind with specific cytosolic proteins during their rapid vectorial transcellular transport from the sinusoidal to the canalicular pole of the hepatocyte. In rat liver cytosol, we have identified in vivo binding to a 3α-hydroxysteroid dehydrogenase (3α-HSD), a 36 kD monomeric oxidoreductase. This enzyme functions as a reductase in vivo catalyzing the metabolism of bile acid precursors at the 3-one position while still retaining the ability to bind bile acid. Co-incubation of bile acids with indomethacin, a nonsubstrate competitive inhibitor of 3α-HSD, resulted in displacement of bile acids from isolated hepatocytes to media. Co-infusion of indomethacin with tracer glycocholate lead to delayed biliary excretion of the bile acid associated with decreased binding to 3α-HSD. These experiments suggest that bile acids interact with the 3α-HSD in vivo thereby retaining them within the cytosol from where they are rapidly excreted by the canalicular transporter. This multifunctional enzyme is also capable of reducing the mutagenic potential of polyaromatic hydrocarbon carcinogens by its dihydrodiol dehydrogenase activity (DDD). Recent cDNA cloning of rat hepatic 3α-HSD/DDD demonstrated significant homology to a family of monomeric NADP(H) reductases. The gene is predominately expressed in the liver and intestine consistent with its role in xenobiotic metabolism and bile acid cytoplasmic binding. Induction of bile acid synthesis or increased hepatic bile acid flux has no effect on steady state mRNA levels.

Using a similar approach, a unique high affinity bile acid binding protein has been purified and recently cloned from human liver cytosol. This protein expresses bile acid dissociation constants of 10- to 100-fold lower

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than the rat and only catalyzes DDD but has no 3α-HSD activity. Human bile acid binder is a 323 amino acid protein with significant sequence homology to the human chloredecone reductase and rat hepatic 3α-HSD. Future studies will focus on determining the physiological role of this protein in bile acid transport and its potential role in regulation of bile acid metabolism.

Dr. Martin Carey summarized new data on secretion and physical-chemical fate of cholesterol in bile. Both the hepatobiliary movement and physical-chemical states of cholesterol (C) in bile can be conceptualized as involving precanalicular events and postcanalicular events. The major rate-limiting step is probably translocation of C across the canalicular plasma membrane (CPM) of the hepatocyte. This membrane domain is unusual in that the CPM has an unusually "stiff" lipid structure, being highly enriched in C and sphingomyelin. The mechanism(s) of transfer of hepatocyte C presumably from endoplasmic reticulum (ER) membranes to CPM is (are) unknown. Apparently, at very high rates of exogenous bile salt (BS) flux, phospholipid-C vesicles may be involved as lipid output into bile under these conditions is sensitive to colchicine inhibition. Because most BS movement is from sinusoidal to canalicular membranes (98%) as part of the enterohepatic circulation with only 2% initiating in intracellular membranes (de novo BS synthesis) and because submicellar BS monomers can bind and translocate C in concentrations about its maximum aqueous solubility $\approx 10^{-8}$ M, diffusive flux of BS-C heteroaggregates may play a role in transcellular movement. Although not yet tested, it is conceivable that the hepatocyte's nonspecific lipid transfer protein (sterol-carrier protein-2) could promote transfer of biliary cholesterol molecules from cytoplasmic membranes to the CPM. With respect to biliary specific phosphatidylcholine (PC) molecular species, submicellar BS concentrations have been shown to stimulate PC transfer from ER to CPM via the hepatic specific PC-transfer protein. Further, there is recent kinetic evidence that translocation of PC to the outer leaflet of CPM may be mediated by a PC "flippase." In the canalicular lumena, several physical-chemical approaches have revealed vesicles but not micelles. On the basis of the 100 kDa monomeric BS transporter and Nernstian principles down an inside-negative (−30−−40 mV) potential, canalicular bile salt concentrations are most likely to be in the 1–2 mM range and therefore monomeric. Recent evidence suggests that vesiculation of the outer layer of the CPM by BS monomers may be instrumental in PC and C secretion as vesicles. A plausible scenario is that following canalicular secretion, ionized bile salt monomers partition preferentially into the luminal monolayers of canalicular membranes. Because these highly charged molecules cannot "flip-flop" to the inner monolayer leaflet, they buildup a critical pressure in the outer leaflet which results in vesiculation. This would target biliary lecithin molecular species in the outer canalicular leaflet, as lecithin molecules would be more "fluid" than other phospholipids, particularly sphingomyelin. In fact, the monomeric bile salt-hemimembrane vesiculation theory would create a potential void on one side of the membrane and this in itself might be sufficient to induce PC "flip-flop" from the inner leaflet to the outer leaflet of the canalicular membrane. The physical-chemical state that results from interactions of bile salt molecules and vesicles depends on the molar ratio of bile salt to vesicle (PC + C) lipids, the dehydration effects of the post-canalicular biliary tree that elevate BS concentrations above their critical micellar concentration, and the influence of time. The intermediate phases formed in the transition sequence from vesicles to mixed micelles are predicted by the appropriate phase diagram at high dilution and have been modeled in vitro employing quasielastic light scattering spectrometry. A reinterpretation of micellar shape and growth from this work as well as from more refined laser-light scattering, neutron scattering, and cryotransmission EM of model biles suggests that micellar growth involves "worm-like" rod-shaped micelles. Recent model studies of bile at high solute and high concentrations formed from vesicles with low C to PC ratios have shown dissolution of vesicles into supersaturated micelles, which is then followed by relaxation to the equilibrium state with the appearance of a new population of C-rich vesicles with a C/PC ratio of 2:1 or greater. Similar C enrichment can occur without vesicle dissolution by virtue of the 4-fold greater transfer of PC molecules to micelles compared with C molecules by virtue of the differential solubilizing power of mixed micelles for each lipid. Concomitantly, the more hydrophobic lecithins of a mixed molecular species (human bile, soy bean, egg yolk, etc.) preferentially partition into vesicles while the least hydrophobic species (least saturated) partition into mixed micelles. Micellization and increased solute concentration of bile render gallbladder bile with supermicellar C contents more unstable compared with dilute hepatic bile. Nucleation and cholesterol crystal growth in both model and native biles is an attempt of the system to drastically minimize its free energy. Using a model bile with a high BS to PC-C ratio we have followed the nucleation process employing state-of-the-art physical-chemical approaches and observed within 5–10 h the formation of large vesicles and/or fused/agglomerated vesicles which are followed by growth of long filamentous C crystals. The filamentous crystals undergo a series of transformation in habit to helices, coils, and tubes which then reopen to form classic C monohydrate plates. Preliminary synchrotron X-ray diffraction and density gradient centrifugation suggest that the filamentous crystals are anhydrous C and that hydration is quite slow in both model and native biles.

Dr. Robert L. Hamilton presented evidence that supports the two-step model of core lipidation of apoB in nas-

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cent VLDL assembly by the endoplasmic reticulum (ER) of hepatocytes. The two-step model originates from two independent observations. First, immunoperoxidase data showed that apoB exists in the rough endoplasmic reticulum (RER) in the absence of a lipid staining particle of VLDL size whereas triglyceride particles of VLDL size in the smooth endoplasmic reticulum (SER) lacked apoB immunostaining consistent with a two-step assembly process (C. A. Alexander, R. L. Hamilton, and R. J. Havel, *J. Cell Biol.* **69:** 241-263. 1976). Second, Drs. Verne N. Schumaker and John Elovson proposed the two-step model at the March, 1992 Deuel Conference on Lipids. These investigators suggested that HepG2 cells lack the second step of triglyceride particle formation perhaps because they lack SER membranes as determined by electron microscopy (R. N. Thrift, T. M. Forte, B. E. Cahoon, and V. G. Shore, *J. Lipid Res.* **27:** 236-250. 1986 and R. L. Hamilton and P. E. Fielding, unpublished observations). Therefore, HepG2 cells secrete apoB-containing particles of HDL and LDL size and density that are produced by the first step in the RER. In addition, Dr. Elovson has found apoB-containing particles of HDL and LDL density in rat liver microsomes consistent with the two-step model (abstracts of Council on Arteriosclerosis, 65th Meeting, AHA, p. 54). The two-step model suggests that a small core lipid-containing, apoB-rich particle is synthesized and released into the cisternal space of the RER, and that a separate triglyceride-rich particle of VLDL size (i.e., 400-600 Å diameter) is formed independently of apoB in the SER and/or the transition compartment, the RER-SER junction.

To test this hypothesis, Dr. Hamilton developed a novel and rapid procedure to isolate large amounts ($\sim$100 mg protein/10 g rat liver) of intact strips of RER from rat liver by modifying the calcium chloride precipitation technique of J. B. Schenkman and D. L. Cinti (*Methods Enzymol.* **52:** 83-89. 1978). In collaboration, Dr. Sandra K. Erickson measured organelle markers in this novel RER fraction. High recoveries of RER enzymes glucose-6-phosphatase, ACAT, and DGAT, and very low recoveries of HMG-CoA reductase (SER), 5' nucleotidase (plasma membranes), succinate INT dehydrogenase (mitochondria), and LDL-receptor (endosomes) were found. Sodium carbonate treatment of this novel RER fraction released three different lipid particles from the cisternal contents. The first floated at d $<$ 1.002 gm/ml, contained little or no apoB, was triglyceride-enriched, and appeared in negative stains as the same size as nascent Golgi VLDL. This may be the particle predicted from the immunoperoxidase study that appeared to be formed independently of apoB in the SER or in the RER-SER junction. Two smaller particles ($\sim$200 Å diameter) floated in sucrose gradients at densities of about 1.024 gm/ml and 1.054 mg/ml. Both of these particles contain large amounts of apoB as determined by Western immunoblotting and by negative staining and both had electron lucent cores indicative of the presence of triglycerides and cholesteryl esters. Preliminary chemical analyses of these two small apoB-containing particles by colleague Dr. Richard J. Havel showed that both contained cholesteryl esters and triglycerides. The prediction is that these small primordial apoB-containing particles from the RER fuse with the triglyceride-rich particles from the RER-SER junction to form a nascent VLDL particle. Fusions between apoB-containing serum LDL and VLDL-sized synthetic microemulsion particles have been shown to occur in vitro (J. S. Parks, J. A. Martin, F. L. Johnson, and L. L. Rudel, *J. Biol. Chem.* **260:** 3155-3163. 1985).

The two-step model of apoB core lipidation in nascent VLDL assembly predicts that the first step may be dissociated experimentally from the second step in intact livers. Previous studies showed that apoB-containing particles of HDL size and density are synthesized and secreted by normal rat livers perfused for prolonged periods (5-6 h) in the absence of exogenous FFA (M. Fainaru, T. E. Felker, R. L. Hamilton, and R. J. Havel. *Metabolism.* **26:** 999-1004. 1977). Furthermore, prolonged perfusions of cholestatic rat livers under the same conditions showed that little VLDL accumulated whereas large amounts of apoB appeared in perfusates in particles of LDL and HDL size and density. Cholestatic livers may be metabolically similar to HepG2 cells with respect to apoB secretions, i.e., the second step is impaired. The two-step model of apoB core lipidation in the endoplasmic reticulum of hepatocytes may explain many observations of VLDL secretion.

Dr. Vishwanath Lingappa discussed the biogenesis of apoB as an example of the regulation of polypeptide chain translocation across the ER membrane, the earliest event in the secretory pathway. In addition to the well-recognized signal sequence peptide and "anchor" sequences, additional sequences known as "stop transfer sequences" have been identified which are involved in translocation across the ER membrane via an aqueous, protein-containing channel. The sequences can be viewed as ligands that interact with receptor proteins to effect targeting to the ER membrane, translocation across it, or termination of translocation, resulting in integration into the membrane bilayer. Thus, multiple signal, stop-transfer, or anchor sequences occurring in register may "stitch" domains of a protein into the appropriate transmembrane orientation through repetitive, sequential cycles of channel assembly and dissembly in the ER membrane. More recent work was described suggesting that, in addition to these constitutive forms of translocation, some processes can be regulated. The biogenesis of amino terminal lengths of apoB and the discrete sequences that direct this may be an example of a regulated form of translocation and, in this regard, may resemble the cases of other complex proteins such as the scrapie prion pro-

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tein and the multi-drug resistance P-glycoprotein.

Dr. Verne Schumaker described the structure of the lipoproteins secreted by HepG2 cells after transfection with plasmids encoding truncated apoB molecules or after release of the growing polypeptides with puromycin. Using isopycnic banding and sedimentation velocity methods, the radii of these small, dense spherical particles were found to be a linear function of apoB size from apoB-25 to apoB-83 and including apoB-100. Correcting for the thickness of the monolayer by extrapolation of the line, the lipoprotein core circumference was calculated, and gave a value of about 1 Å of circumference per kDa of apoB. The suggestion was made that apoB size determines the core size and that the mechanism might be that the apoB with attached phospholipid attempts to surround a triglyceride droplet in the inner layer of the ER, succeeding in doing so when the polypeptide chain is released from the ribosome and the two ends of apoB can meet, automatically detaching the newly formed lipoprotein from the inner ER leaflet.

Dr. Sven-Olof Olofsson discussed pulse-chase experiments with apoB made by HepG2 cells. Cells were pulse-labeled with [^{35}S]methionine and incubated with cycloheximide to block further chain elongation and the nascent polypeptides released by puromycin. The products were isolated from either the microsomal lumen or the medium by sucrose gradient ultracentrifugation, the apoB was immunoprecipitated, and the precipitates were separated by SDS-PAGE. During the chase, the nascent polypeptides, after reaching 100–200 kDa banded in the gradient with HDL densities and were gradually converted into longer polypeptides on less dense particles ending up with full-length apoB-100 on LDL- and VLDL-sized particles. Other experiments indicated that the apoB-100 bound to the ER membrane was not used for lipoprotein assembly but was degraded. The formation of LDL-VLDL particles occurred in ER regions with high diacylglycerol transferase activity and it was postulated that apoB-100 has three intracellular paths. If co-translationally lipidated, it will translocate to the ER lumen; if not, it will be degraded. If it is not fully lipidated, immature particles may be formed which are also degraded.

Dr. Fred Kern, Jr. reported on his studies to determine the effects of dietary cholesterol on cholesterol metabolism and cholesterol gallstones (GS) in humans. The hypothesis that patients with GS respond to dietary cholesterol differently than controls was tested. Eight volunteer women with GS and eight matched control subjects were studied twice: first on their regular diets containing 220 mg/day of cholesterol (C), and again after feeding an additional 5 eggs/day for 15–17 days, mean C intake 1280 mg/day. Dietary C intake, plasma lipids, C absorption, C synthesis, biliary lipid composition and secretion, and bile acid kinetics were measured by established methods suitable for ambulatory patients. Plasma lipids were found to be normal and did not change in either group. Biliary lipid composition and secretion did not differ on the low C diet, but on the high C diet, biliary C secretion increased in GS subjects only. On the low C diet, C absorption was slightly less and C synthesis was 44% greater ($P < 0.001$) in GS. On the high C diet, C absorption and synthesis decreased in both groups, but synthesis remained higher in the GS group. On the low C diet the bile acid fractional turnover rates were faster and the pool sizes were smaller in GS patients. Bile acid synthesis did not differ. In control subjects neither bile acid synthesis nor pool size changed on the high C diet, but in GS both synthesis and pool size decreased ($P < 0.05$ for both). Turnover rates did not change.

Dr. Kern concluded that GS patients synthesize more C than control subjects and when fed a high C diet, secrete more C into bile and synthesize less bile acid. The decrease in bile acid synthesis and pool size contrasts sharply with the response in subjects without GS. These alterations in hepatic processing of dietary C by GS subjects probably cause the secretion of bile supersaturated with C, a necessary first step in the formation of GS.

Dr. Lawrence Rudel discussed his work using African green monkeys as a primate model for the dietary cholesterol-induced hypercholesterolemia and gallstone formation seen in humans. Groups of animals were fed diets with low, moderate, or high cholesterol content in order to induce plasma cholesterol concentrations that mimic this in humans at low, moderate, or high risk to premature CHD. In each of the animals in the study, periodic measurements of plasma lipids, lipoproteins, and apolipoproteins were made. Dietary cholesterol induced higher LDL cholesterol and apoB concentrations, more cholesteryl ester per LDL particle, and a higher LDL/HDL ratio, all changes positively associated with atherosclerosis. The percentage of dietary cholesterol absorbed from the intestine was inversely associated with the level of cholesterol in the diet and was positively correlated to plasma total cholesterol concentration. The mechanism for this effect was hypothesized to be associated with decreased bile acid availability as reflected in reduced production by the liver. Dietary cholesterol previously had been shown in these animals to result in a 40% decrease in the activity of cholesterol 7α-hydroxylase (C7H), the rate-limiting enzyme in the conversion of cholesterol to bile acid. The hepatic mRNA abundance for C7H was found to be 60–70% lower in animals fed dietary cholesterol in this study. Bile acid binding drugs reduced plasma cholesterol and LDL concentrations rapidly and dramatically concomitant with a marked decrease in the percentage of intestinal cholesterol absorption. Bile acid secretion rates by the liver in intact animals were near 200 mmol/h. Bile acid secretion was then measured in the presence of an interrupted enterohepatic cir-

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culation (bile fistula animal), and the value was maintained at 20 mmol/h and was unable to increase in response to the absence of bile acid return to the liver. Taken together, these findings suggest that C7H in this species is very sensitive to down-regulation by dietary cholesterol. This response appears to represent an attempt by the liver to limit cholesterol return from the intestine by decreasing bile acid availability and thus, cholesterol absorption. Dr. Rudel hypothesized that individuals who can more effectively limit bile acid availability will be able to maintain lower LDL cholesterol concentrations in the face of a dietary cholesterol challenge.

Dr. John Deitschy proposed that the steady-state level of plasma LDL cholesterol is determined by four separate parameters of LDL metabolism. These include: *1)* the LDL-cholesterol production rate (Jt), *2)* maximal receptor-dependent transport (Jm), *3)* the affinity of the LDL molecule for the LDL receptor (km), and *4)* the magnitude of receptor-independent LDL transport (P). Dietary lipids, including cholesterol and the various fatty acids, appear to alter the steady-state plasma LDL-cholesterol level by varying only the LDL-cholesterol concentrations that are related to the amount of cholesterol added to the diet. In male hamster, for example, the plasma LDL-cholesterol level will increase from approximately 25 mg/dl to approximately 80 mg/dl as the dietary cholesterol content is increased from very low levels to 0.12%. Measurement of the absolute rates of cholesterol absorption and total body cholesterol synthesis demonstrate that these changes are associated with an increase in the net delivery of cholesterol into the body. There is an associated marked increase in the level of cholesteryl esters in the liver suppression of the rate of hepatic cholesterol synthesis, suppression of hepatic LDL receptor activity, and an increase in the LDL-cholesterol production rate.

When the level of cholesterol in the diet is kept constant and various specific fatty acids are fed, the effects on these parameters of LDL metabolism depend very much on the structure of the specific fatty acid. The 12:0, 14:0, and 16:0 saturated fatty acids bring about further suppression of LDL receptor activity and a marked increase in the LDL-cholesterol production rate. These changes result in an elevation of the plasma LDL-cholesterol concentration but, paradoxically, the steady-state level of cholesteryl esters in the liver is markedly lowered. The 18:1 monounsaturated fatty acid, in contrast, significantly elevates the cholesteryl ester level in the liver, restores hepatic LDL receptor activity, lowers the LDL-cholesterol production rate, and markedly lowers the plasma LDL-cholesterol concentration. These striking effects are essentially lost when a second double bond is placed in the fatty acid molecule, i.e., the 18:2 fatty acid, or when the double bond in the 18:1 compound is converted to the *trans* configuration. Similarly the 6:0, 8:0, 10:0, and 18:0 fatty acids are essentially biologically neutral with respect to the parameters of LDL metabolism. When the absolute rates of cholesterol synthesis and cholesterol absorption were measured in animals receiving the 14:0 and 18:1 fatty acids, it was found that there was no change in net delivery of cholesterol to the liver under the circumstances where there had been marked changes in LDL receptor activity and LDL-cholesterol production rates.

Thus, these studies are consistent with the view that there are essentially two different mechanisms for regulating hepatic LDL receptor activity and the circulating levels of LDL-cholesterol. The first mechanism is mediated by changes in net cholesterol delivery to the liver and is articulated through a putative regulatory pool of sterol in the liver cell that is in equilibrium with the cholesteryl ester pool. Thus, the greater the net delivery of sterol to the liver, the higher is the putative regulatory pool and cholesteryl ester pool and the greater is suppression of LDL receptor activity. In contrast, fatty acids exert their marked regulatory effects independent of changes in net cholesterol balance across the liver. It is postulated that this regulation is articulated through the effects of the fatty acid on the distribution of cholesterol between the cholesteryl ester pool and the putative regulatory pool. In contrast, the 18:1 monounsaturated fatty acid shifts this equilibrium toward the cholesteryl ester pool and so lowers the amount of sterol in the regulatory pool and enhances hepatic receptor activity. In any event, it is clear from these studies that regulation of hepatic receptor activity, and therefore plasma LDL-cholesterol levels, is achieved by dietary cholesterol and dietary fatty acids in two fundamentally different ways.

Dr. Murray Heimberg proposed that any component of VLDL can theoretically be rate limiting for the formation and secretion of VLDL by the liver. To evaluate a potential role for cholesterol (C), he reduced the hepatic C/cholesteryl ester (CE) pool in the rat with the HMG-CoA reductase inhibitor lovastatin, and observed a significant inhibition of VLDL secretion in vitro and in vivo. This effect, moreover, was reversible by dietary C or by addition of LDL to the medium in vitro. These observations have been supported by other investigators using experimental animals, and have been suggested by studies in man. Since C reversed the effects of the reductase inhibitor, it was considered that excess dietary C, in addition to the well-established role of free fatty acids (FFA), might stimulate VLDL secretion. This was observed to be the case. In the rat, output of VLDL by the isolated perfused liver was increased in proportion to the content of C in the diet, and was related to the CE content of the liver. The effects of FFA and C on secretion of VLDL, moreover, appeared to be additive. Dietary C increased the secretion of all the VLDL lipids and apolipoproteins (B-100, B-48, E). It is of particular interest that dietary C

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increases hepatic triglyceride (TG) concentration and secretion in the rat, in addition to the expected rise in CE. Cholesterol induced the secretion of increased numbers of VLDL particles in which the core ratio of TG/CE was altered. This may be a major mechanism by which dietary C may induce hypertriglyceridemia, as well as hypercholesterolemia. To evaluate potential mechanisms by which dietary C increased the net quantity of TG in the rat, we investigated aspects of FFA metabolism in vivo and in the isolated perfused liver in vitro. Using [1-^{14}C]oleate, it was observed that C feeding stimulated esterification of FFA to TG (and CE) in vivo and in vitro. Oxidation (ketogenesis and ^{14}CO formation) of [^{14}C]oleate by the perfused liver was reduced by dietary C. Thus, C feeding stimulated synthesis of TG and decreased oxidation of FFA. Furthermore, activity of mitochondrial carnitine palmitoyltransferase, a potential rate-limiting step in fatty acid oxidation, is reduced by C feeding. Another potential mechanism by which C may increase TG concentration in VLDL and liver is by stimulation of the rate of de novo synthesis of fatty acids. Dr. Heimberg's preliminary experiments with ^3H_2O in intact rats, indeed, suggests this to be the case. The biochemical and molecular mechanisms by which dietary C affects hepatic metabolism of FA are the subjects of current and future work in this laboratory. Dr. Heimberg suggests that C induces such changes because TG is required in the structure of the VLDL in this species to transport excess C/CE from the liver to the plasma.

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