A NATO-sponsored Advanced Research Workshop (ARW 990-83) on “Coordinate Regulation of Cholesterol Metabolism” was held from October 1-3, 1984, in Santa Fe, New Mexico. The Giovanni Lorenzini Foundation of Italy was also a sponsor. The organizing director was Dr. Ajit Sanghvi, University of Pittsburgh; the co-directors were Drs. Giovanni Galli, University of Milan, Italy, and Terence J. Scallen, University of New Mexico. Drs. David Kritchevsky, The Wistar Institute, Philadelphia, and George Vahouny, George Washington University, Washington, D.C., served on the advisory committee.

The last 10 years have provided considerable information on the regulation of cholesterol metabolism via lipoprotein receptor action in various types of cells. A significant body of information about in vitro changes in the activities of cholesterol-metabolizing enzymes has also developed. Changes in the state of phosphorylation of HMG-CoA reductase by experimental manipulations has been shown to be correlated with changes in the enzyme activity. The experimental systems used in these studies have used broken-cell preparation, intact hepatocytes, and whole animals. Similar but less extensive information has recently been gathered for acyl-coA:cholesterol acytransferase (ACAT) and cholesterol 7α-hydroxylase, the other two major enzymes responsible for cholesterol metabolism in the liver cell. There is debate as to whether phosphorylation/dephosphorylation of these enzymes plays a role in vivo and, if it does, the extent of its significance. However, those who suggest enzyme phosphorylation/dephosphorylation as a possible in vivo mechanism for regulating cholesterol flow within the cell suggest it as only a short-term regulatory phenomenon; changes in protein synthesis are required for the long-term.

This NATO Workshop was organized to review the more recent information pertinent to the regulation of liver cholesterol metabolism. The following is a summary of each session in the order in which they appeared on the program.

**Cholesterol Absorption**

This session reviewed the various factors that affect cholesterol absorption in humans. The first speaker, Professor Luigi Barbara from the University of Bologna, Italy, provided an up-to-date view on the effect of bile acid structure and function on biliary cholesterol secretion in humans. The next speaker, Professor Maurizio Ponz de Leon from the University of Modena, Modena, Italy, discussed the various factors affecting cholesterol absorption in humans. He presented an overview of several methods that have been devised for studying cholesterol absorption in humans and stated that all have drawbacks and most require the administration of labeled compounds. The available information indicates that the amount of cholesterol in the diet is a crucial factor in influencing the absorbed fraction.

Similarly, the presence of bile acids in the upper intestine represents *conditio sine qua non* for cholesterol absorption, since patients with bile duct fistula are unable to absorb cholesterol. The relative importance of individual bile acids in this regard has been investigated in the last few years. These investigations suggest that trihydroxy bile acids stimulate cholesterol absorption, while the absorbed fraction decreases after dihydroxy bile acid feeding, both in animals and in humans. In rats, this effect seems to be related to the stimulatory effect of cholic acid and its conjugates on the intestinal esterification of cholesterol, whereas in humans the data are still unclear. It does not seem, however, that the stimulatory effect of trihydroxy bile acids on cholesterol absorption can be attributed solely to an effect on intestinal cholesterol esterase activity.

Other factors affecting cholesterol absorption include dietary fat (and probably other nutrients), small bowel transit time, and thyroid hormones. Recent studies indicate that acceleration of small bowel transit time, induced by pharmacological means, is fol-
lowed by a significant reduction of dietary cholesterol absorption. Prolongation of transit time, on the other hand, has no effect in this regard. Hyperthyroidism is frequently associated with marked reductions of the absorbed fraction. Normalization of the thyroid function in these patients leads to an increase toward normal in the absorption of dietary cholesterol. But hyperthyroidism does not seem to have a consistent effect on cholesterol absorption.

Dr. Ponz de Leon concluded by saying that several physiological factors probably concur in affecting cholesterol absorption in humans. Thus, the absorbed fraction may represent the net balance between factors that enhance cholesterol absorption (for example, the amount of dietary cholesterol, the cholic acid pool size, the abundance of fat) and factors that inhibit absorption, (such as dihydroxy bile-acid pool size and acceleration of small bowel transit time).

The Effect of Nutrition and Pharmacologic Agents

In this session, Dr. David Kritchevsky from the Wistar Institute, Philadelphia, Pennsylvania, discussed the relationship between diet and cholesterol metabolism. He mentioned that studies of this nature have been limited to the study of dietary effects on serum or plasma cholesterol. Although there have been some studies of the influence of diet on other aspects of cholesterol metabolism (synthesis, excretion, degradation, and distribution), these have been few in number.

More recently, several studies have investigated the effect of dietary protein on plasma cholesterol levels. These studies indicate that, in general, protein from animals is more cholesterolemic than protein from plants. Cholesterol turnover is slower in rabbits fed casein and their fecal excretion of bile acids is less than in rabbits fed soy protein. Dr. Kritchevsky suggested that one factor influencing the metabolic activity may be these proteins’ ratio of lysine to arginine.

Saturated fat is usually more cholesterolemic than unsaturated fat; however, the effects of fats on cholesterolemia seem to be influenced by triglyceride structure and fatty acid content. Thus cocoa butter, a saturated fat rich in stearic acid, is less cholesterolemic in humans and less atherogenic in rabbits than palm oil.

In recent years, dietary fibers have often been in the news. Dietary fiber is a generic term covering a number of substances with different structures and physiological effects. Studies indicate that gelling fibers, such as pectin or guar gum, are hypocholesterolemic but that water-holding fibers, such as cellulose or bran, are not. In fact, in rats, cellulose increases total body cholesterol.

Dr. Kritchevsky concluded his talk by remarking that although it is relatively easy to study diets in which only one component is altered, a more accurate picture of dietary effects will require a thorough study of dietary interactions.

The next speaker in this session, Dr. Mitchell Cayen from the Ayerst Laboratories, Princeton, New Jersey, discussed the various types of pharmacologic agents and their effect on cholesterol metabolism and serum cholesterol levels. He divided the various antihyperlipidemic agents into several major categories as follows: 1) clofibrate and its analogues, such as bezafibrate, gemfibrozil, and fenofibrate; 2) nicotinic acid and its pro-drugs and analogues; 3) anion-exchange resins, such as cholestyramine, colestipol, and polidexole; 4) inhibitors of sterol absorption, such as sitosterols, sucrose polyester, and nomycin; 5) inhibitors of HMG-CoA reductase such as compactin and mevinolin; 6) other drugs, such as probucol.

The three major determinants of plasma cholesterol levels are: 1) dietary cholesterol, 2) cholesterol synthesis, and 3) excretion of neutral sterols and bile acids. Pharmacological agents can lower serum cholesterol by interfering with any of these. Dr. Cayen suggested that a number of factors should be considered in assessing the mode of action of any antihyperlipidemic agent, including the mechanism of lipid exchange between lipoprotein classes, the effect on apoprotein components, and the effects of regulatory activities, such as HMG-CoA reductase, cholesterol 7α-hydroxylase, lipoprotein lipase, and LCAT. The current research on pharmacologic agents indicates that the pharmacokinetics of the absorbable antihyperlipidemic agents varies markedly, and the elimination half-lives of these drugs do not correlate well with their therapeutic efficacy.

Dr. Cayen mentioned two other classes of pharmacologic agents that can alter circulating levels of cholesterol and triglycerides. These are the β- and α-adrenergic blockers. In general, most β-adrenergic blockers tend to reduce high density lipoprotein (HDL) cholesterol and the ratio of HDL/total cholesterol, and tend to elevate plasma triglycerides. On the other hand, α blockers increase HDL cholesterol and the HDL/total cholesterol ratio, and tend to reduce plasma triglycerides.

Lipoprotein Interactions

This session was addressed by Dr. Bryan Brewer from the National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland. He described in detail the most recent advances in the study of the structure, function, and metabolism of plasma lipoproteins.

Human plasma lipoproteins are a polydisperse collection of particles containing lipid and proteins. Currently, 13 well-characterized apolipoproteins are known. These apoproteins (apo) have multiple functions such as: 1) cofactors for enzymes (apo C-II, lipoprotein lipase and apo A-I, lecithin cholesterol acyltransferase); 2) ligands for interaction with cellular receptors; 3) exchange proteins for neutral lipids;
and 4) structural constituents of lipoprotein particles.

Studies on apoprotein biosynthesis indicate that apo A-I and apo A-II are synthesized as preproapoproteins, whereas apo E, apo C-I, apo C-II, apo C-III, and apo A-IV are synthesized as preproteins.

The metabolism of triglyceride-rich lipoproteins has been shown to consist of two cascades that involve lipoproteins containing apo B. The first apo B cascade involves the stepwise metabolism of chylomicrons secreted by the intestine and is designated apo B-48. Shortly after secretion, the triglyceride-rich chylomicrons undergo lipolysis and are sequentially reduced to remnants having the density of VLDL and then IDL. The chylomicron remnants are removed by an apo E-mediated receptor uptake by the liver.

The second apo B cascade involves hydrolysis of triglyceride-rich VLDL secreted by the liver. These lipoprotein particles contain apo B-100, a larger molecular form of apo B secreted by the liver. The chylomicrons on VLDL also undergo hydrolysis by lipoprotein lipase. These are then serially converted to lipoproteins with the density of IDL and finally, LDL. LDL contains principally apo B-100 and interacts with receptors on the plasma membrane of liver and peripheral cells, ultimately resulting in endocytosis and catabolism of the LDL particles.

HDL, which contains primarily apo A-I and apo A-II, are synthesized directly by the liver and intestine as well as by the transfer of lipid and apolipoprotein constituents from triglyceride-rich lipoproteins during lipolysis. It has been proposed that the major function of HDL is the transfer of cholesterol from peripheral cells to the liver by the process termed “reverse cholesterol transport.”

Dr. Brewer concluded his lecture by remarking that the molecular defects in patients with dyslipoproteinemias are now recognized to be due to defects in apolipoproteins, enzymes, and lipoprotein receptors. He suggested that the elucidation of the molecular defects in these patients now provides the opportunity to develop more specific therapy for these patients.

Methodology

Dr. Enrica Bosisio from the University of Milan, Milan, Italy, gave an extensive discussion of a number of methods currently used to assess the activities of three principal enzymes in cholesterol metabolism: HMG-CoA reductase, cholesterol 7α-hydroxylase, and ACAT.

HMG-CoA reductase activity can be determined by both radioisotopic and nonradioisotopic methods. The radioisotopic method that is used most often is based on the measurement of labeled mevalonate enzymatically formed from 14C HMG-CoA.

The nonradioisotopic method that measures the amount of reduced CoA lacks sensitivity, and the one based on the measurement of oxidized NADPH formed during the reaction requires the solubilization and partial purification of the enzyme. Because of the nature of these methods, they have limited usefulness.

Another nonisotopic procedure that measures the amount of mevalonate synthesized from nonradioactive substrate by selected ion monitoring in crude extracts of microsomal suspensions has been recently described. This method has the advantage of specificity and can be particularly useful when liver microsomes have low activity or when the amount of microsomal protein available is limiting.

The determination of cholesterol 7α-hydroxylase activity is complicated because the enzyme utilizes microsomal cholesterol as the substrate. As with HMG-CoA reductase, the activity of this enzyme may also be monitored using radioisotopic as well as nonradioisotopic procedures. In the former, the 7α-hydroxylase activity is estimated by measuring the extent of conversion of radioactive cholesterol into labelled 7α-hydroxycholesterol. This method is complicated and requires standardization of the form in which the labeled cholesterol substrate is added because equilibrium of this exogenously added cholesterol with the endogenous cholesterol is dependent on both the concentration and the manner in which the substrate cholesterol is added. A correction estimation of the enzyme activity can be achieved by measuring the absolute amount of 7α-hydroxycholesterol formed. In this connection, there are now three methods available: one double-isotope-derivative dilution procedure and two mass-spectrometry procedures which use a selected ion-monitoring technique to measure the mass of 7α-hydroxycholesterol. One of the latter methods requires the synthesis of deuterated standards and thin-layer chromatography of the reaction products; the other mass-spectrometric method avoids these steps and is simpler.

ACAT activity can be determined from the incorporation of labeled fatty acids or acyl-CoA esters into cholesteryl esters using endogenous cholesterol as cosubstrate, or by using labeled cholesterol.

The use of fatty acyl-CoA esters is preferable to the use of labeled cholesterol, because the former has better water solubility. Where labeled cholesterol is used, it must be added with solvents or detergents. This, in turn, generates questions of equilibration and dilution with the endogenous “substrate pool,” the size of which is difficult to establish and is rate-limiting.

Fatty acyl-CoA esters can be replaced by the addition of fatty acids plus CoA plus ATP. In this instance, however, the resulting enzyme kinetics appear to be different under different experimental conditions. The ACAT has a high specificity for oleate or oleyl-CoA.

Cellular Aspects

Section on Methyl Sterols

This session was divided into five subsections. The first section opened with a lecture by Dr. Tatu
Miettinen from the University of Helsinki, Helsinki, Finland. He indicated that human serum lipoproteins contain five major methyl sterols, including lanosterol and its demethylation products and two dimethylsterols and two monomethylsterols in unesterified form. In contrast to lathosterols, methyl sterols are not esterified in serum by LCAT, which suggests that esterified serum methyl sterol, composed mainly of monomethyl sterols, are formed by hepatic ACAT and released in association with VLDL into the blood. Furthermore, squalene and methyl sterols exhibit up to sixfold diurnal variation, while the esterified methyl sterols show very little variation.

Further studies with methyl sterols have indicated that a short-term cholesteramine treatment, which increases cholesterol and bile acid synthesis, and serum-free lathosterols and methyl sterols, gives a negligible increase in esterified methyl sterols. On the other hand, squalene feeding, which also stimulates cholesterol and bile acid synthesis, increases the free and esterified methyl sterols and the hepatic ACAT activity. Reduction of cholesterol synthesis by fasting lowers free methyl sterols but markedly increases the esterified methyl sterol fraction. These findings indicate that esterified methyl sterols in serum may reflect intrahepatic ACAT activity and, less consistently, hepatic production of methyl sterols.

Dr. Kurt Einarsson discussed the studies in his laboratory at the Karolinska Institute on the regulation of hepatic enzymes that affect cholesterol metabolism in human liver. In 11 gallstone patients, HMG-CoA reductase activity averaged about 80 pmol/min/mg protein. The enzyme activity assayed in the presence of 50 mM NaF was only 15% to 20% of the activity in its absence. Preincubation of microsomes prepared in NaF with E. coli alkaline phosphatase resulted in a tenfold increase of enzyme activity. The activated enzyme could be inactivated by incubation of microsomes with Mg-ATP. Inclusion of protease inhibitors in the homogenizing media had no apparent influence on the activation or inactivation properties of the enzyme. Treatment with chenodeoxycholic acid produced a 40% reduction of HMG-CoA reductase activity, whereas cholic acid and ursodeoxycholic acid had no effect.

In this study, seven gallstone patients had a cholesterol 7α-hydroxylase activity averaging 10 pmol/min/mg protein, corresponding to a daily synthesis of about 0.5 mmol bile acids. Cholesteramine treatment of patients resulted in a fourfold higher cholesterol 7α-hydroxylase activity. Dr. Einarsson observed that NaF, Mg-ATP, and E. coli alkaline phosphatase did not influence the 7α-hydroxylase activity in human liver microsomes. He pointed out, however, that the phosphate buffer used in preparing microsomes and in the assay may have partially contributed to the failure of these agents to influence 7α-hydroxylase activity.

In Dr. Einarsson's study, ACAT activity averaged about 4 pmol/min/mg protein in nine gallstone patients. Immediate freezing of the liver biopsy in liquid nitrogen before homogenization and microsome preparation increased the enzyme activity about sixfold, but the mechanism of this effect is not known.

Section on Regulation of Cholesterol Metabolism

The second section was devoted to a discussion of the enzymatic mechanisms of short-term and long-term regulation of cholesterol metabolism. The first enzyme discussed was HMG-CoA reductase. The discussion was opened by Dr. Zafarul Beg from the National Heart, Lung, and Blood Institute, National Institutes of Health, who presented data on the mechanisms involved in the modulation of HMG-CoA reductase activity in rat and human livers. Dr. Beg and his colleagues have established that HMG-CoA reductase activity is modulated by a bicyclic cascade system involving phosphorylation and dephosphorylation of both HMG-CoA reductase and reductase kinase. They have also demonstrated that in intact rats, phosphorylation of HMG-CoA reductase is modulated by glucagon and mevalonolactone due to increased phosphorylation (activation) of reductase kinase. The activity of reductase kinase kinase appears to be modulated in vivo by short-term (20-minute) administration of mevalonolactone, thus providing a mechanism for the regulation of enzymic activities of both reductase kinase and HMG-CoA reductase. In turn, mevalonolactone administration appears to cause a significant inhibition of phosphoprotein phosphatase(s) activity which dephosphorylates both HMG-CoA reductase (activation) and reductase kinase (inactivation). The net effect of this sequence is to increase the steady-state level of the phosphorylated forms of both HMG-CoA reductase and reductase kinase. Both reductase kinase and reductase kinase kinase are cyclic nucleotide independent protein kinases.

The more recent studies by Dr. Beg and colleagues have identified a mechanism for the modulation of HMG-CoA reductase activity by protein kinase C, which is a calcium-activated and phospholipid-dependent protein kinase. Protein kinase C appears to be able to phosphorylate both microsomal insoluble ($M_r \sim 100,000$) and purified soluble ($M_r \sim 53,000$) HMG-CoA reductase. In these studies, the phosphorylation and concomitant inactivation of HMG-CoA reductase was completely dependent on Ca$^{2+}$, phosphatidyl serine, and diolein, which are the known cofactors for protein kinase C activity. The maximal phosphorylation of purified HMG-CoA reductase was associated with the incorporation of 1.05 ± 0.16 mol of phosphate per mole of the native form of HMG-CoA reductase ($M_r \sim 100,000$).

Studies from Dr. Beg's laboratory further show that phorbol 12-myristate 13-acetate (PMA), a tumor-promoting phorbol ester, stimulates protein kinase C catalyzed phosphorylation of HMG-CoA reductase. These studies demonstrate the possibility of in vivo, protein kinase-mediated mechanisms for the regulation of HMG-CoA reductase activity.
The next speaker in this session was Dr. David Gibson from Indiana University Medical School, Indianapolis, Indiana, who presented his most recent studies on the control of HMG-CoA reductase activity by reversible phosphorylation in rat hepatocytes. Dr. Gibson's studies showed that in isolated, intact hepatocytes, glucagon produces a decrease in expressed reductase activity, total reductase activity, and protein phosphatase activity, while it activates reductase kinase activity. Insulin opposes these changes. Thus, a hormone-sensitive bicyclic system, which regulates HMG-CoA reductase activity in rat liver microsomes, can also be demonstrated in hepatocytes. This is similar to results from studies in intact rats, in which the addition of mevalonolactone to hepatocytes brings about a precipitous fall in expressed reductase activity, probably through inhibition of reductase dephosphorylation. Total reductase activity falls after the acute decline in expressed activity. Dr. Gibson quantified the 97,000 dalton holoenzyme form of reductase by using an SDS gel electrophoretic separation of hepatocyte microsomal proteins and an immunoblot transfer technique. These studies indicated that mevalonolactone and 25-hydroxycholesterol accelerate the degradation of the native reductase in hepatocytes incubated with these agents for 3 hours. Insulin treatment diminished the rate of degradation of the 97,000 dalton band, as well as the parallel loss of total reductase activity.

Further studies by Dr. Gibson showed that microsomal reductase that is pretreated with ATP and reductase kinase and then incubated with homogeneous, calcium-activated, neutral, thiol protease (calpain) from rat liver generates both a membrane-bound 62,000 dalton immunoreactive fragment of the native reductase, and a soluble reductase species (53,000 daltons). Qualitatively, the dephosphorylated native enzyme is cleaved to similar products but at a much slower rate. These findings suggest that phosphorylation of reductase not only acutely diminishes expressed reductase activity but also accelerates reductase degradation.

The last speaker in this series was Dr. Fausto Hegardt from the University of Barcelona, Barcelona, Spain, who presented his studies on the regulation of cholesterol biosynthesis by microsomal protein phosphatases. Dr. Hegardt has solubilized the reductase phosphatase activity from microsomes and has purified three different microsomal reductase phosphatases by column chromatography using DE-52, phosphocellulose, aminohexyl Sepharose 4B, and BioGel A. The molecular weights of the phosphatases designated $E_1$, $I_1$, and $I_2$ are 75,000, 180,000, and 90,000 daltons, respectively. The pH optimum for these isolated phosphatase activities range between 6.0 and 6.5. Treatment with 80% ethanol irreversibly denatured the three phosphatases. The three microsomal reductase phosphatases were inactivated by the most common protein phosphatase inactivators including pyrophosphate, nucleoside triphosphates, and also by the reductase substrates, NADPH and HMG-CoA. All three phosphatases were found to dephosphorylate $^{32}$P-labeled HMG-CoA reductase (subunit $M_r ~ 52,000$) with the concomitant activation of the enzyme. All three also dephosphorylated phosphorylase $a$ and glycogen synthase D. Phosphorylase $a$, glycogen synthase D, and HMG-CoA reductase were noncompetitive substrates for all three microsomal phosphatases, suggesting an independent regulation of glycogen and cholesterol metabolisms.

Section on ACAT Regulation

The third part of this session considered the regulation of ACAT activity. Dr. Kathleen Gavey from the University of New Mexico, Albuquerque, New Mexico, provided data from her laboratory showing that ACAT activity may be regulated by the mechanism of phosphorylation/dephosphorylation.

The enzyme ACAT, which esterifies cholesterol in rat liver cells, is thought to play a role in maintaining unesterified cholesterol homeostasis within the cell. Increasing the amount of cholesterol available to ACAT increases the rate of cholesterol ester biosynthesis, and it has been proposed that this is a regulatory mechanism for this enzyme. Dr. Gavey found that ACAT in microsomes prepared from rat liver could be inactivated and reactivated by conditions that favor dephosphorylation and phosphorylation, respectively. The enzyme was inactivated in the presence of $Mg^{2+}$ and was reactivated by a protein kinase partially purified from microsomal membranes. A soluble, partially purified phosphoprotein phosphatase that activated microsomal HMG-CoA reductase was found to inactivate ACAT. The effect of this protein was blocked by potassium fluoride. These observations provide the bases for Dr. Gavey's suggestion that ACAT and HMG-CoA reductase are oppositely regulated by phosphorylation and dephosphorylation.

The next speaker, Dr. Kostas Mitropoulos from the Medical Research Council, Hammersmith Hospital, London, England, discussed the data from his laboratory suggesting that the transfer of cholesterol from hepatic plasma membranes to endoplasmic reticular membranes could be a major factor modulating the activity of the ACAT enzyme. Dr. Mitropoulos presented data showing that plasma membrane vesicles in the rat liver microsomal fraction can be donors of cholesterol for the membrane vesicles that contain ACAT. Consequently, preincubation of the microsomal fraction results in a time- and temperature-dependent increase in the ACAT activity. This flow of cholesterol to membrane vesicles can be increased in the presence of phosphatidylcholine cholesterol liposomes (1:1, mol/mol) or it can also be diverted to phosphatidylcholine liposomes. The transfer of cholesterol between microsomal and artificial membrane vesicles follows first-order kinetics in the concentration of cholesterol in the donor vesicles. There is a progressive increase...
in the rate of transfer of liposomal cholesterol to the microsomal vesicles with an increasing concentration of liposomal cholesterol. On the other hand, the rate of transfer to the ACAT substrate pool increases with increasing concentration of cholesterol in the plasma membrane vesicles, and the rate of transfer of plasma membrane cholesterol to phosphatidylcholine liposomes increases with increasing concentration of liposomes present in the incubation mixture.

Preincubation of the microsomal fraction in the presence of cytosol also shows an increased transfer of plasma membrane cholesterol to the ACAT substrate pool or to phosphatidylcholine liposomes. Furthermore, rates of transfer of liposomal cholesterol to the microsomal vesicles and then to the ACAT substrate pool are higher in the presence of cytosol than in its absence. This information raises the question of the relative importance of this mechanism that involves a transfer of cholesterol between plasma membrane and endoplasmic reticular membranes and the mechanism of phosphorylation/dephosphorylation in the regulation of ACAT activity.

Section of Bile Acid Synthesis

The fourth section in this session was devoted to a discussion of the regulation of bile acid synthesis. The first paper was given by Dr. Warren Diven from the University of Pittsburgh, Pittsburgh, Pennsylvania, who presented data showing the possible role of phosphorylation/dephosphorylation in the modulation of 7α-hydroxylation activity. Cholesterol 7α-hydroxylase catalyzes the rate-limiting first reaction of the pathway leading to the biosynthesis of bile acids. This enzyme is a mixed-function oxidase requiring the participation of a species of cytochrome P-450, NADPH, NADPH cytochrome C reductase, and molecular oxygen.

When rat liver microsomal membranes are incubated in 20 mM Tris HCl buffer, a time-dependent loss in cholesterol 7α-hydroxylase activity takes place. This loss of activity is not observed in 200 mM phosphate buffer, or if 50 mM NaF is added to the Tris buffer. The loss of activity occurs more rapidly if E. coli alkaline phosphatase is included in the incubation. Enzyme activity lost with such a preincubation can be partially restored by incubating inactivated microsomes with ATP (Mg) and more completely restored if a cytosolic protein fraction is included in the incubation with ATP (Mg).

Recent studies in Dr. Diven’s laboratory indicate that cholate extraction of rat liver microsomes and chromatographic purification of the extract on DEAE Sephadex and Sephadex G-200 yields four separate phosphatases. These phosphatases possess a degree of substrate-specificity. For instance, the DEAE breakthrough fraction was found to be least effective in inactivating microsomal 7α-hydroxylase and most effective in activating HMG-CoA reductase and inactivating ACAT. Other fractions possessed intermediate levels of substrate specificities. While the phosphatase-induced inactivation of rat liver 7α-hydroxylase has been demonstrated, the possibility of this being an indirect effect has not been ruled out.

Dr. Kjell Wikvall from the University of Uppsala, Uppsala, Sweden, discussed the role of various cytochrome P-450 fractions in the biosynthesis of bile acid and their regulation. Several cytochrome P-450 fractions which are active in hydroxylations of C 27-steroids involved in bile acid biosynthesis have been purified from rat, rabbit, and pig liver microsomes and mitochondria. These fractions possess a high degree of specificity for hydroxylations in the various positions of C 27-steroids. Of particular interest are the fractions that promote cholesterol 7α-hydroxylation, 12α-hydroxylation, and 26-hydroxylation. The 7α-hydroxylation of cholesterol is subject to metabolic control, and the 12α-hydroxylation has been ascribed a regulatory role. The 26-hydroxylation is catalyzed by a specific liver mitochondrial cytochrome P-450.

Studies with a reconstituted enzyme system consisting of NADPH cytochrome C reductase and a species of cytochrome P-450 shows that the 7α-hydroxylation is catalyzed by a cytochrome P-450, which has a shorter half-life than the one which catalyzes 12α-hydroxylation. This reconstituted enzyme system is activated by cysteine, dithiothreitol, reduced glutathione, and thioredoxin, whereas glutathione disulfide inactivates the enzyme. Two other proteins isolated from cytosol also exert regulatory effects on this reconstituted 7α-hydroxylation system. One of these proteins stimulates the enzyme activity in the presence of reduced glutathione or thioredoxin, whereas the other protein inhibits the 7α-hydroxylation activity.

The last paper in this section was given by Dr. Ingemar Björkhem of Huddinge Hospital, Huddinge, Sweden. He discussed the mechanism of regulation of phosphatidic acid phosphatase by phosphorylation-de-phosphorylation and the relation between this enzyme and cholesterol 7α-hydroxylase.

Evidence that there is a relation between the turnover of plasma triglycerides and the synthesis of bile acids in humans has accumulated. Patients with hypertriglyceridemia often have an increased production of bile acids. Cholestyramine treatment and bilary drainage, which increase the formation of bile acids, also increase the rate of formation of triglycerides. Treatment with chenodeoxycholic acid, which inhibits bile acid synthesis, is followed by a decrease in biosynthesis of triglycerides. The nature of the link between biosynthesis of triglycerides and bile acids has not yet been elucidated.

Studies by Dr. Björkhem in which he has investigated the effect of lymph flux and bile acids on the hepatic phosphatidic acid phosphatase indicate that although substrate availability may be most important, the soluble phosphatidic acid phosphatase seems to be rate-limiting for the biosynthesis of tri-
glycerides in the liver. Studies concerning the regulation of the activity of this enzyme show that incubation with ATP inhibited the reaction and this inhibition was reversible by alkaline phosphatase. After ethanol treatment, it was still possible to modulate the activity of phosphatic acid phosphatase with these effectors.

Lymphatic, as well as biliary, drainage and treatment with cholestyramine stimulated the soluble phosphatic acid phosphatase in rat liver. The microsomal phosphatic acid phosphatase was affected about the same as the soluble enzyme, whereas the microsomal acyltransferase, believed to be a noregulatory enzyme in triglyceride biosynthesis, was unaffected. Dietary cholesterol decreased the soluble phosphatic acid phosphatase activity toward microsomal-bound substrate about 50%, but did not decrease the activity toward micellar substrate. Also, in some experiments it was found that chenodeoxycholic acid markedly inhibited the soluble phosphatic acid phosphatase activity.

These findings suggest that the enterohepatic circulation of bile acids, as well as the flow of chylomicrons to the liver, may be important for the regulation of phosphatic acid phosphatase activity.

For comparisons, Dr. Björkhem also studied the effects of the above treatments on the rate-limiting enzymes in the biosynthesis of cholesterol and bile acids in parallel. Both cholesterol 7α-hydroxylase and HMG-CoA reductase were stimulated less by the lymphatic drainage than by treatment with cholestyramine. In contrast, the phosphatic acid phosphatase was, in general, stimulated more by the lymphatic drainage than by treatment with cholestyramine.

Section on the Role of Carrier Protein and Cellular Transport

The last section in this session was devoted to a discussion of the role of carrier protein and cellular transport in cholesterol metabolism. Dr. Terence J. Scallen from the University of New Mexico, Albuquerque, the first speaker in this section, reviewed studies that showed the role of sterol carrier protein in cholesterol biosynthesis and utilization.

The rat liver cytosol contains two proteins that are required for the microsomal conversion of squalene to cholesterol. One of these proteins, termed "sterol carrier protein," is required for the enzymatic conversion of squalene to lanosterol by liver microsomal membranes. This protein is identical to the supernatant protein factor purified by Dr. Konrad Bloch. The second of the two proteins, sterol carrier protein, (SCP2), activates enzymatic conversions of intermediates between lanosterol and cholesterol by liver microsomes. Recently, Dr. Scallen has shown that in the presence of SCP2 there is a striking increase in the conversion of exogenously added cholesterol into cholesterol ester by rat liver microsomes. He has suggested that SCP2 may be the means by which endogenous cholesterol, or dietary cholesterol, is delivered to ACAT in the endoplasmic reticulum of rat liver in vivo.

Studies by Dr. George Vahouny from George Washington University, Washington, D.C., on the role of SCP2 in specific aspects of the intracellular transfer of lipids and in sterol utilization for sterol synthesis have used cellular fractions of rat adrenocortical tissue. These studies indicated that SCP2, but not the fatty-acid-binding protein, stimulates cholesterol release from droplets with cholesterol-rich lipids in a stoichiometric manner. Cholesterol thus released from lipid droplets in the presence of SCP2 can be transferred to mitochondria and will accumulate at the inner membrane of aminoglutethimide-treated mitochondria, which are inhibited from further utilizing the sterol. Release of the inhibition causes levels of steroid production higher than those in control mitochondria.

SCP2 also affects a transfer of cholesterol from the outer face of the outer membrane of mitochondria to the inner membrane site of cytochrome P-450sec.

Dr. Vahouny also described the immunoreactive SCP2-like activities that have been found in cytosolic fractions of adrenal and other tissues that metabolize or transfer large amounts of sterol (ovary, liver, and intestine tissues). He added that in the adrenal, the stimulatory effect of the cytosolic fraction on cholesterol transfer and mitochondrial steroid synthesis is completely lost when the cytosol is pretreated with anti-SCP2 IgG.

In Dr. Ajit Sanghvi's Pittsburgh laboratory, studies have been carried out on the role of sterol carrier protein2 in bile acid synthesis. These studies indicate that microsomal incubations that contained SCP2 exhibited enhanced formation of 7α-hydroxycholesterol under various experimental manipulations. Further experiments have also shown that SCP2 is able to increase 7α-hydroxylase activity regardless of the source of substrate cholesterol (cholesterol in the form of serum lipoprotein or of phosphatidylcholine/cholesterol liposomes). Microsomes treated with anti-SCP2 IgG lose about 80% of their ability to synthesize 7α-hydroxycholesterol.

This information, developed in three different laboratories, suggests that SCP2 could represent the intracellular transport protein for cholesterol translocations and, where applicable, for cholesterol metabolism; for example, bile acid synthesis, esterification, and possibly other hydroxylations.

Summary

Dr. David Gibson provided a concise summary of the conference and concluded by saying that each investigator present possessed one or two pieces of the puzzle of cholesterol metabolism in the liver. He added that this workshop had brought these individuals into one place in an attempt to fit the pieces together.