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Clinical Methods in Study of Cholesterol Metabolism

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Contents

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Introduction

Cholesterol is one of the structural components of all cellular and intracellular membranes in the human body. Of the 140 g or so of cholesterol present in the entire body, less than 7% is present in the plasma [90, 286], and clinically this small fraction of the total body cholesterol is perhaps the most important. Patients with high levels of plasma cholesterol tend to have a much greater risk of heart attacks as compared to those with relatively lower levels of plasma cholesterol [232], and evidence accumulated in recent studies suggests that reduction of plasma cholesterol may reduce the risk of death from myocardial infarction [6, 289]. Although there is general agreement that it would be better to lower plasma levels of cholesterol, there is no unanimity of opinion as to the best way to achieve this objective.

It is generally believed that increased levels of plasma cholesterol are associated with increased rates of deposition of cholesterol in tissues including the site of atherosclerotic lesion: the arterial intima. Smoking and hypertension are other factors which influence the rate of progress of atherosclerotic changes [119, 233]. Attempts to decrease plasma cholesterol by diet or drugs are based on the hope that reduction in the rates of deposition of cholesterol may decrease both the size of the lesions as well as the risk of myocardial infarction. The observations that the cholesterol deposits in the tendons and subcutaneous tissues reduce in size when plasma cholesterol concentrations are decreased tend to support this hope. However, the relationships, if any, between the atherosclerotic lesions and the deposits of cholesterol in tendons or other body pools are not known. There is no information whether the rates of deposition in the atherosclerotic lesions or its removal from those lesions parallel the changes in the cholesterol pools in tendons or other body tissues. It is not even known that any observed reduction in mortality and morbidity from complications of atherosclerosis is mediated through an actual reduction in the cholesterol deposits in atherosclerotic lesions.

Despite extensive studies on cholesterol metabolism over the last few decades, a large number of questions still remain unanswered. The methods

for reliable studies in man have become available only recently and they have remained difficult and time-consuming. Extensive data from animal studies cannot be indiscriminately extrapolated to man. There is a pressing need for more work in man and simpler methods are badly needed to facilitate this.

The primary objective of this book is to introduce various methods currently available for the study of cholesterol metabolism in man. The theoretical as well as practical aspects of these methods are given in adequate details for those interested in the study of cholesterol metabolism. No attempt has been made to deal extensively with all aspects which bear directly or indirectly on the chemistry of these methods. However, there should be no difficulty in the understanding and the execution of these methods if details (and sometimes references) given here are followed.

It is recognized that knowledge of metabolism of plasma lipoproteins (and of the methods involved) is also necessary for a proper perspective on cholesterol metabolism. Adequate recent reviews on this subject are available and therefore it has not been dealt with in this small volume. It is, however, recommended that the student of cholesterol metabolism should be thoroughly familiar with the metabolism (and structure) of plasma lipoproteins.

Chapter I. Chemistry of Cholesterol

Historical Background

Cholesterol was discovered as the major component of gallstones in the 18th century, and *De Fourcroy* [107] was among the first to prepare large quantities of a crystalline substance from extracts of human gallstones in the latter half of the century. *Chevreul* [83, 84] showed that the substance remained unchanged after boiling with potassium hydroxide, and he coined the word 'cholesterine' (from Greek 'cole', bile; 'steros', solid). This substance was then identified in human and animal bile and also in the human brain. It was later identified in hen's eggs and gradually was recognized as a normal constituent of all animal cells. In 1859, 'cholesterine' was identified as an alcohol by *Berthelot* [34] and he prepared esters of it. Later, in 1896, *Hürthle* [220] isolated cholesterol esters CE) from serum, and in 1910 *Windaus* [504] showed that the cholesterol in the atheromatous lesions was present chiefly as esters.

The credit for the elucidation of the structure of cholesterol goes mainly to *Windaus* and his associates [503–506]. In 1919, he proposed a tentative formula for cholesterol which was subsequently changed in 1932 to the one now accepted (fig. 1).

Related Steroids

The term 'steroid' is applied to compounds containing perhydrocyclopentaophenanthrene carbon skeleton. The four closed carbon rings are identified as A, B, C, and D, and the numbering of carbon atoms in the molecule is continued from the rings to the side chain (fig. 1). Cholesterol has a hydroxyl group (OH) at carbon position 3 and a double bond between carbons 5 and 6 [41, 140].

Although cholesterol is the most abundant steroid in the mammalian tissues, small quantities of other compounds related in structure to choles-

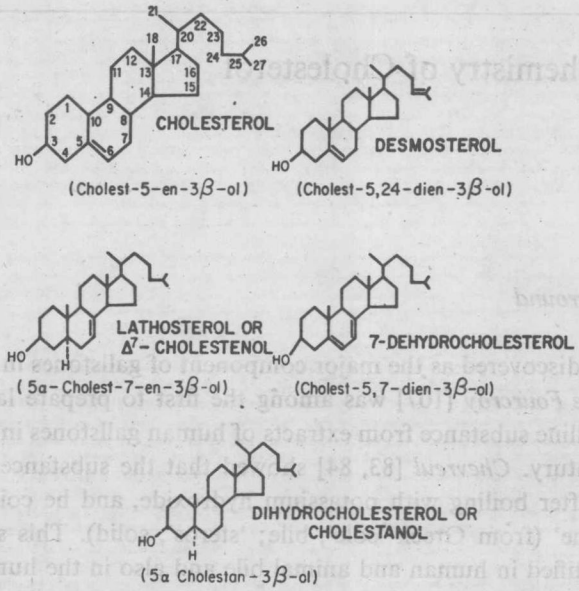

Fig. 1. Structure of cholesterol and some of the related steroids present in animal tissues.

terol are also present. A precursor of vitamin D, 7-dehydrocholesterol, has a structure almost identical to that of cholesterol in that it has only one additional double bond at carbons 7 and 8 (fig. 1), and it can be converted to vitamin D by ultraviolet (UV) irradiation of skin [35, 52].

Dihydrocholesterol or cholestanol which differs from cholesterol and lathosterol in that it has no double bond in its ring structure and Δ7-cholestenol or lathosterol (which has a double bond between carbons 7 and 8) (fig. 1) are also present in trace quantities [136–138, 298]. The bile acids, cholic and chenodeoxycholic acid, are the two primary bile acids derived from cholesterol [31, 44]. Cholesterol is also converted to a large number of steroidal hormones, such as progesterone, androgens, estrogens, adrenal cortical hormones, etc. [42, 196, 516].

Comparable steroidal compounds present in plants are generically known as 'phytosterols'. Campesterol (C28), stigmasterol (C29), and β-sitosterol (C29) are the most abundant of the plant sterols (fig. 2). Since these sterols can be absorbed, albeit only to a small extent [170, 390], they are also found in trace amounts in human tissues.

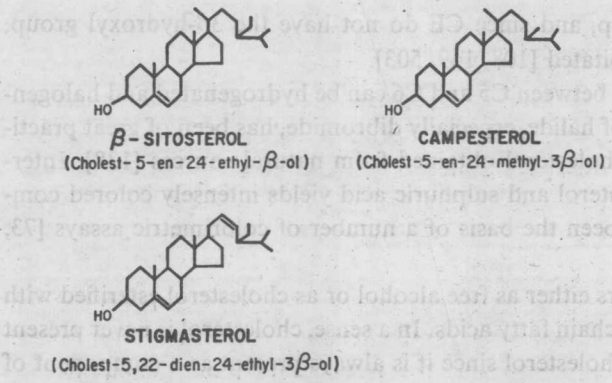

Fig. 2. Structure of the major plant sterols present in human diet.

Physical and Chemical Properties of Cholesterol

Both the ring structure and the aliphatic side chain of cholesterol are nonpolar. The 3-β-hydroxyl group is the only polar group in the cholesterol molecule. It is, therefore, relatively insoluble in water and quite soluble in organic solvents. Its solubility in ethanol is much less than in diethyl ether [41]. Cholesterol is solid at room temperature. Its melting point is 149.5–150 °C [137, 138]. It can be distilled under high vacuum and can also be sublimed. Since there are several asymmetric carbon atoms, solutions of cholesterol exhibit optical rotation which can aid in the identification and in ascertaining the purity of cholesterol preparations [41]. When crystallized from anhydrous organic solvents, it forms triclinic needles, and when crystallized from 95% alcoholic solution it separates as monohydrate, rhomb-shaped triclinic plates, which lose water at 70–80 °C [41].

A variable fraction of cholesterol is present in the human body as CE [220, 347]. The fatty acids forming CE are long chain fatty acids generally containing 16–20 carbon atoms. A smaller quantity of shorter or longer chain fatty acids may also form CE in the body. The esterification of cholesterol with fatty acids occurs at the 3β position. Since the only polar group present in the free cholesterol is lost on the formation of esters, CE are essentially nonpolar.

The glycoside digitonin, the saponins tigonin and gitonin, and the alkaloid tomatine, interact with the 3β-hydroxyl group of cholesterol and precipitate with cholesterol. The reaction is specific for sterols containing

the 3β-hydroxyl group, and since CE do not have the 3β-hydroxyl group, they cannot be precipitated [108, 149, 503].

The double bond between C5 and C6 can be hydrogenated and halogenated. The formation of halide, especially dibromide, has been of great practical use in purifying cholesterol obtained from natural sources [138]. Interaction between cholesterol and sulphuric acid yields intensely colored compounds and this has been the basis of a number of colorimetric assays [73, 262, 391].

Cholesterol occurs either as free alcohol or as cholesterol esterified with one of the many long chain fatty acids. In a sense, cholesterol is never present in the body as 'free' cholesterol since it is always present as a component of macromolecular complexes called lipoproteins. The 'free' cholesterol implies that it is not esterified. The bulk (approximately 80–90%) of cholesterol present in most tissues is free sterol [90]. Red blood cells (RBC) and the nervous system contain little if any of CE, while cholesterol in plasma and adrenals is present predominantly as esters [4, 50].

Other sterols, such as 7-dehydrocholesterol, dihydrocholesterol (cholestanol), etc., are present in tissues in small but variable quantities. Their concentrations in plasma are negligible; thus, their contamination does not significantly affect the quantitation of cholesterol in plasma. However, in some experiments involving the determination of specific activity (SA) of cholesterol after administration of radioactive precursors, it may be necessary to remove these (radioactive) contaminants and purify cholesterol.

Chapter II. Isolation, Purification, and Estimation of Cholesterol

The most common source of samples containing cholesterol in clinical practice is plasma or serum. For experimental studies also, the plasma and its various lipoprotein fractions are the most important sources of information. However, in some experiments, it may be necessary to analyze diet, feces, bile, and tissues for their cholesterol content.

Collection, Handling, and Storage of Samples

Collection of Blood

The concentration of plasma cholesterol is not markedly altered during the absorptive phase, but the triglycerides are significantly increased after a fatty meal. For that reason, the subjects are advised not to take anything but water for 14–16 h before the collection of blood samples. It is necessary to stop the medication known to affect the lipid metabolism for a few weeks before taking the blood samples. Other drugs taken by the subject should also be noted.

Plasma volume and concentrations of certain blood constituents change with the change in posture. When a subject stands upright from a recumbent position the plasma volume decreases, and the reverse happens when the subject changes from an upright to a recumbent position. The change in plasma volume has been attributed to the outward movement of fluid due to an increase in hydrostatic pressure [135, 490, 515]. *Stoker et al.* [451] reported that 15 min after healthy subjects changed from a recumbent to an upright position, the plasma cholesterol concentration increased by 12.5%. These findings have been confirmed by *Tan et al.* [470] who also showed that the concentration of plasma triglycerides increased by 12.4% when subjects changed from a lying to a standing position. Subjects assuming a sitting instead of an upright position showed similar but smaller changes in the concentrations of plasma lipids. Since the magnitude of these changes is similar to those observed after treatment with some hypolipidemic agents, these studies indicate the necessity of standardizing the postural position for

collection of blood. Ideally, blood samples should be collected 15 min after the subject has maintained a sitting position. In any case, posture should be constant for a given subject during a given study, and one should wait at least a fixed interval of a few minutes in that posture before blood is collected.

Application of a tourniquet for 5 min or longer can also cause comparable increases in the concentration of plasma cholesterol [235, 342], although its application for less than a minute does not significantly affect plasma lipids [470]. In collecting blood samples, the tourniquet should be released immediately after the needle is placed inside the vein and the blood samples should be withdrawn after a few seconds.

Blood samples should be drawn from an antecubital vein or from some other convenient arm vein. Vacutainer tubes containing solid EDTA are convenient for collection of 1–25 ml of blood. The instructions for collections are generally supplied with the system. Use of solid EDTA as an anticoagulant eliminates dilution which could occur with tubes containing solutions of EDTA in saline. EDTA not only serves as an anticoagulant but also has the additional advantage of chelating divalent metallic ions that promote the autooxidation of lipids [366]. Other anticoagulants, such as heparin, could also be used [185, 198]. However, the blood should be kept below 10 °C and the processing of the sample should not be unduly delayed.

Samples can also be drawn directly in syringes wetted with an anticoagulant. Immediately after sampling, the blood should be transferred to tubes containing an anticoagulant. Prior to the transfer of blood into test tubes, the needles must be removed to prevent hemolysis. Once filled, the contents of the tubes must be mixed promptly by inverting 7–8 times. Mixing must be thorough but not vigorous. Blood is then placed in a refrigerator (4 °C) or in an ice bath pending separation of plasma [103, 320].

Normal blood contains an enzyme (lecithin: cholesterol acyltransferase or LCAT) which esterifies plasma free cholesterol (FC) [153]. Thus, the ratios of plasma FC to esterified cholesterol may change significantly during storage of plasma at room temperature. If blood samples contain radioactive cholesterol then the SA of plasma CE may also change as a result of the activity of this enzyme. The SA of FC may change due to exchange of FC between RBC and plasma lipoproteins [187]. Both the activity of the enzyme and exchange of FC between plasma and RBC decrease to insignificant levels at low temperatures [434]. Therefore, it is necessary not only to keep the samples at or about 4 °C, but that the plasma should be separated from cells in a refrigerated centrifuge as soon as possible after collection of the

blood. The activity of LCAT could be inhibited by adding reagents such as *p*-hydroxymercuribenzoate, iodoacetate, *N*-ethyl maleimide, etc. [153], or by keeping the samples chilled. The latter also reduces the risk of autooxidation of plasma lipids.

Separation of Plasma

The tubes containing blood are centrifuged at 4 °C in a refrigerated centrifuge for 15–20 min at 1,600 *g* [192, 319]. Plasma is promptly removed from the sedimented cells using a Pasteur pipette with a rubber bulb, taking care that plasma is not drawn in the bulb. It is transferred into a screw cap tube and stored in the dark at 4 °C after adding inhibitors of LCAT enzyme, if necessary.

For more details on collection and handling of blood, the reader is referred to the *Manual of Laboratory Operations for Lipid and Lipoprotein Analysis* [266].

Separation of Plasma Lipoproteins

Except in rare instances (type I and type V hyperlipoproteinemia), fasting samples of plasma do not contain chylomicra. If chylomicra are present, they will float to the top when stored overnight in a refrigerator. Alternatively, they can be removed by centrifugation at 26,000 *g* for 30 min or 100,000 *g* for 10 min [264].

The isolation of different plasma lipoproteins can be carried out by a number of procedures. The most commonly used techniques for isolating major lipoprotein classes is preparative ultracentrifugation and is described briefly below. For more details, the reader is referred to several excellent reviews [132, 194, 264].

Preparative Ultracentrifugation of Plasma Lipoproteins

The procedure as developed by *Lindgren* and his associates [263, 264] is described below and is based on the use of the Beckman Preparative Ultracentrifuge, Model L2–65.

The plasma samples used for separation of the lipoproteins should either be fresh or stored at 4 °C for less than 5 days. The samples must not be frozen. If stored at 4 °C, plasma samples should be equilibrated at room temperature for at least 45 min before centrifugation.

Preparative ultracentrifugation involves raising the density of 'the background' solutions of lipoproteins either by adding solid salt or by mixing salt solutions of known density. The most commonly used salts are NaCl

for isolating low density and a mixture of NaCl and NaBr for isolating high density lipoproteins. All salt solutions should contain EDTA 100 mg/l.

The lipoproteins are classified according to the density of the background solution from which they are floated. Very low density lipoproteins (VLDL) are floated at d <1.006 g/ml, intermediate density lipoproteins (IDL) at d = 1.006–1.019 g/ml, low density lipoproteins (LDL) at d = 1.019–1.063 g/ml, and high density lipoproteins (HDL) at d = 1.063–1.21 g/ml.

Generally, the VLDL, LDL, and HDL are removed sequentially from the same plasma sample. Chylomicra (Svedberg flotation [Sf] >400) are rarely present in fasting samples, and if necessary they can be removed in a preliminary step by centrifuging at 100,000 *g* for 10 min at 23 °C in a 60 ti rotor or by centrifuging in a Beckman SW41 ti rotor at 1.6×10^6 *g*/min. VLDL are separated as follows. 4 ml plasma is transferred into a 6-ml cellulose nitrate tube and overlayered with exact amount of 0.196 *m* NaCl to yield a final volume of 6 ml. After centrifugation at 40,000 rpm for 18 h at 18 °C, VLDL are quantitatively removed in the upper 1-ml fraction. The second 1-ml fraction is taken to check the background density. The 4-ml bottom fraction is mixed thoroughly with a glass stirring rod and the contents quantitatively transferred to a new preparative tube. 2 ml of salt solution (0.196 *M* NaCl, 0.5052 *M* NaBr, d = 1.0435 g/ml, $N_D26 = 1.3405$) is then added and the total volume is adjusted to exactly 6 ml. This yields a salt background density (before centrifugation) of d = 1.019 g/ml. After centrifugation as above, IDL are quantitatively obtained in the top of the 1-ml fraction and another 1 ml is taken as background. The bottom 4-ml fraction is mixed thoroughly and the contents quantitatively transferred to a new preparative tube. 2 ml of the salt solution (0.196 *M* NaCl, 2.433 *M* NaBr, d = 1.1816 g/ml, $N_D26 = 1.3644$) is then added, and the total volume is adjusted to exactly 6 ml using the same salt solution. The centrifugal conditions are the same as above. After the run is complete, LDL (d = 1.063 g/ml) are quantitatively obtained in the top of 1 ml fraction and another 1 ml is taken as a background. The bottom 4 ml is then throroughly mixed again and transferred to a new preparative cellulose nitrate tube. 2 ml of 0.196 *M* NaCl, 7.572 *M* NaBr (d = 1.4744 g/ml, $N_D = 1.4120$) is added and mixed. This yields a salt background (before centrifugation) of d = 1.216 g/ml. Centrifuge at 40,000 rpm for 24–26 h. The total HDL are removed in the top of 1 ml fraction and 0.5 ml of the fraction is taken as reference background.

When removing the samples from centrifuge, great care should be exercised to avoid any abrupt movement of the rotor or of the tubes. A