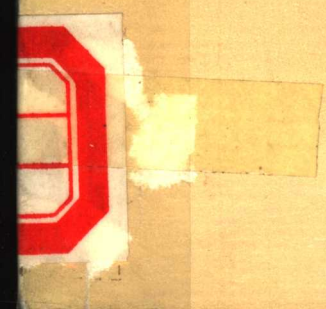
Clinical Biochemistry

Contemporary Theories and Techniques

VOLUME 2

Edited by
HERBERT E. SPIEGEL



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Preface

The purpose of this volume is to again present the ideas and philosophies of scientific leaders in the many collateral fields related to clinical chemistry. The editor has tried to retain the individual style of each contributor rather than to present a highly stylized, regimented volume. It is hoped that this unorthodox method of presentation will still reflect good and scholarly science.

A further intent of this volume is to supplement the existing textbooks of clinical chemistry and biochemistry by providing areas of emphasis such as coagulation chemistry, inborn errors of metabolism, and biochemistry of aging, which are not emphasized in standard clinical chemistry texts. Individual details are de-emphasized in favor of overall concepts and philosophy, where possible. Speculation and interpretation by the contributors has been encouraged, with the idea that these may be stimulating to the reader.

This book is a collection of scientific essays that is designed hopefully to acquaint the reader with the wide spectrum of sciences that is called clinical chemistry.

Herbert E. Spiegel

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Acknowledgments

I would like to acknowledge the contributions and professionalism of all those who participated in assembling this volume. My secretary, Mrs. Joan Marks, again has proven to be a valuable and valiant assistant. Finally, to the members of my family, especially my wife Joanne, I express my special thanks for the gifts of patience and concern.

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1

Biochemistry and Clinical Significance of Lipoprotein-X

SHESHADRI NARAYANAN

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I. INTRODUCTION

Lipoprotein-X (LP-X) is an unique lipoprotein that has acquired clinical significance in that it is a marker for obstructive jaundice, since it is present in the sera of patients. It is also seen in the plasma of subjects suffering from a rare inborn error of metabolism—the deficiency of lecithin–cholesterol acyltransferase (LCAT).

LP-X has been characterized as a low-density lipoprotein from its behavior in the ultracentrifuge. It is distinct chemically, immunochemically, and electrophoretically from other lipoprotein species. Indeed, because of

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these distinct characteristics, it was named LP-X to set it apart from other lipoprotein species, such as LP-A and LP-B. The biochemistry of LP-X, in terms of its metabolism and role, can best be understood by gaining an insight into its unique chemical and structural properties and its diagnostic significance.

II. CHEMISTRY OF LP-X

Our knowledge of the chemistry of LP-X is based upon studies conducted with highly purified preparations. Procedures for purification of LP-X from cholestatic plasma entail separation from other lipoprotein fractions. This can be done by exploiting the density differences of the lipoprotein fractions in an ultracentrifuge. Thus, the very low-density lipoprotein fractions (VLDL) can be made to float by applying plasma beneath a layer of buffer with a density of 1.0055 g/ml, and centrifuging at a high speed (105,000 *g*) for 22 hr (*1,2*). The heavier lipoprotein fractions containing LP-X can be isolated by precipitation of the low-density lipoprotein fraction with heparin and manganese. Further manipulative steps include fractionation with ethanol, the adjustment of the density of the supernatant to 1.063 g/ml with sodium chloride, and ultracentrifugation at 105,000 *g* for 44 hr. LP-X bands in the fraction that has a floatation density of 1.006–1.063 g/ml.

Instead of utilizing sequential ultracentrifugation steps, isolation of LP-X can also be accomplished with zonal ultracentrifugation in a sodium bromide gradient, subsequent to a preliminary ultracentrifugation step to remove VLDL (*3*). Zonal ultracentrifugation procedures are less time-consuming than sequential ultracentrifugation. However, the former, because of the introduced effects of dilution, requires a concentration step by ultrafiltration.

The purity of LP-X isolated by zonal ultracentrifugation procedures is apparently dependent on both the duration and speed of centrifugation. While LP-X was isolated as a single species upon high speed zonal ultracentrifugation at 150,000 *g* for 135 min (*3*), lower speeds permitted fractionation of LP-X into two or three species (*4,5*).

Thus, rate zonal ultracentrifugation at 80,000 *g* for 2 hr results in the resolution of LP-X into two fractions, LP-X_1 and LP-X_2 (*4*). Centrifugation at the same speed for approximately 72 hr results in the appearance of another LP-X fraction, LP-X_3 (*5*).

LP-X can also be purified by chromatography on hydroxyapatite since both VLDL and β-lipoprotein are retained on the adsorbent, permitting early elution and separation of LP-X (*3*).

Zonal ultracentrifugation procedures without prior ethanol fractionation yield LP-X preparations that are not completely free from contaminating low-density lipoprotein, apolipoprotein-B(APO-B). Each of the LP-X fractions (LP-X_1, LP-X_2, and LP-X_3) have been purified to homogeneity by ethanol fractionation and zonal ultracentrifugation repeated twice in a linear gradient (*5*).

III. ANALYSIS OF LP-X PREPARATIONS

LP-X purified from human and animal cholestatic plasma are similar in chemical composition (*1,6*). When compared to other lipoprotein fractions (VLDL, LP-B, and high density lipoprotein—HDL), LP-X has a unique chemical composition with the phospholipid content higher than any other lipoprotein fraction. Phospholipid constitutes 66.5% of the LP-X molecule (*1,7*). The next major constituent is unesterified cholesterol, which constitutes 22.4%. Cholesterol esters and triglycerides together constitute approximately 5% of LP-X. Protein content is also low, amounting to approximately 6% of the LP-X molecule. The fact that the esterifying capacity of cholesterol is impaired in patients with obstructive jaundice might explain the extremely low levels of cholesterol esters found in LP-X.

LP-X, when fractionated into three species (LP-X_1, LP-X_2, and LP-X_3), still retains the characteristic phospholipid:unesterified cholesterol ratio seen in the native LP-X molecule (*5*). There are slight differences, however, in the amounts of the minor constituents (VLDL, cholesterol esters, and protein) that make up the three species of LP-X. Lecithin is the major and sphingomyelin is the minor phospholipid found in LP-X. The latter constitutes just one-fifth of the concentration of lecithin (*2*). The lecithin:sphingomyelin ratio of LP-X is almost twice that found in the LDL fraction, but is, however, similar to that found in the HDL fraction.

The 14-carbon fatty acid, myristic acid, is the major fatty acid constituting the cholesterol esters found in the three fractions of LP-X—(LP-X_1, LP-X_2, and LP-X_3 (*5*). It is interesting that the enzyme LCAT prefers a phosphatidyl choline donor of LP-X that has 14-carbon fatty acids (*5,6*). Linoleic acid has also been found as a constituent of cholesterol esters. Linolenic and higher unsaturated fatty acids are esterified to make up the triglycerides in the LP-X molecule. Differences in the fatty acid content of linoleic, linolenic and higher unsaturated fatty acids are seen in the LP-X fractions isolated from different individuals. The significance of this variability in the fatty acid content of LP-X isolated from cholestatic plasma of different individuals is not clear.

The bile acid content of LP-X has been reported to be variable, and ranges from 0.14–3% of the LP-X molecule. The major bile acid found in LP-X is the hepatotoxic lithocholic acid (*8*).

The protein moiety of LP-X is made up of apolipoprotein-X (APO-X), which constitutes 60% of the protein. The remainder of the protein moiety is contributed by albumin, which is tightly associated with the apoprotein. Albumin is implicated in the maintainence of the structural integrity of the LP-X molecule.

The amino acid composition of APO-X is unique in that it differs from that found in apoproteins A and B. APO-X has no cystine or cysteine, and its histidine content is very low (*2*). The major N-terminal amino acids found in APO-X are serine and threonine, whereas the major C-terminal amino acid found was alanine. The composition of APO-X is identical to that of apolipoprotein-C (APO-C) obtained from the partially delipidized VLDL fraction in plasma (*9*). Our current knowledge is that both APO-X and APO-C have three different polypeptide chains made up of N-terminal serine and C-terminal alanine, N-terminal threonine and C-terminal valine, and N-terminal threonine and C-terminal glutamic acid (*2*). Both APO-X and APO-C have similar phospholipid to protein ratios.

Both APO-X and APO-C have a great affinity for phospholipid, as is evidenced by the high phospholipid to protein ratio of 11.5 found in LP-X. The ability to bind phospholipid is related to the presence of helical structures in both APO-X and APO-C. The helical structure with its polar and nonpolar halves presents a conformation that is conducive to the binding of phospholipid. The hydrophobic content of the helix determines the amount of phospholipid bound (*6,10*). Differences are seen in the α-helicity of the three fractions of LP-X (*5*). The secondary structure of APO-X (APO LP-X_1 and APO LP-X_3) is similar to human serum albumin, which is a constituent of LP-X. This secondary structure is relatively stable. LP-X_2 and LP-X_3 contain, in addition to APO-C, the apoproteins APO A-1 and APO-E. APO A-1 is an activator of the enzyme lecithin–cholesterol acyltransferase (LCAT) (*11*). APO-E is rich in the amino acid arginine, and appears in the plasma of patients with a deficiency of LCAT and in type III hyperlipidemia (*12*).

The authenticity of these analytical data obtained with purified LP-X preparations has been documented by immunochemical studies (*1,5,6*). The identity of APO-X and APO-C was established in immunodiffusion experiments using antisera to either APO-C or LP-X (*2*). By performing immunoelectrophoresis of APO-X in 1% agar, the presence of three species of LP-X was demonstrated by the formation of three immunoprecipitin arcs with antibody to LP-X (*2*). The fact that LP-X reacts with antisera to albumin, only upon delipidation, is consistent with the tight association of albumin with APO-X. Our knowledge from immunochemical studies on

the structure of LP-X is that APO C-II and albumin are located within the core of the molecule. The reactivity of the three species of intact LP-X ($LP\text{-}X_1$, $LP\text{-}X_2$, and $LP\text{-}X_3$) with antisera to APO C-I and APO C-III suggests that these two apoproteins are on the surface of the LP-X molecule (*5*). Phospholipids are also located on the surface of LP-X, as demonstrated by the denaturation of LP-X on treatment with phospholipase A_2 (*13*).

Studies using electron microscopy (*13*), X-ray small-angle scattering (*14*), and electron paramagnetic resonance (*5*) have provided valuable information on the structure of LP-X. Our knowledge based on these studies reveals that LP-X is heterogeneous and that the three species ($LP\text{-}X_1$, $LP\text{-}X_2$, and $LP\text{-}X_3$) are very rigid particles. This rigidity is due to its unique chemical composition reflected by its high concentration of free cholesterol and the 14-carbon saturated fatty acid. These rigid particles are spherical vesicles with a lipid bilayer of cholesterol and phospholipids in combination with APO-X. A proposed model for LP-X visualizes it as being a spherical particle which is nearly 400 Å in diameter (*10*). In this model APO-C-I and APO-D are on the outer surface of the particle, with albumin, APO-C-II, and APO-C-III located within the inner core. Albumin exists in the inner core in solution without being bound to the phospholipid-free cholesterol bilayer. This model also depicts a part of the APO-X moiety in combination with the polar head of the phospholipids.

IV. PROPERTIES OF LP-X

LP-X aggregates with membrane-bound enzymes. Complexes of LP-X with alkaline phosphatase have been isolated from cholestatic human sera and characterized. Complexes of LP-X with γ-glutamyltranspeptidase, 5′-nucleotidase, and nucleotide pyrophosphorylase have also been demonstrated (*6,15*). Enlargement of the erythrocyte membrane has been noticed in patients with cholestasis. Electron microscopic studies have ascribed the cause of this enlargement as due to the fusion of LP-X vesicles with the erythrocyte membrane (*16*).

V. METHODOLOGY FOR THE MEASUREMENT OF LP-X

A. Electrophoresis

LP-X has a characteristic cathodal mobility on agar gel which lends itself to both qualitative and quantitative assay. By incorporating a drop of a lipid stain, such as sudan black, at the point of application of the sample

to the agar gel, it is possible to visualize the migration of LP-X. The concentration of agar commonly used is 1%, in barbital buffer, pH 8.6. A typical electrophoretic run lasts 90 min. (*6*). A variety of procedures are available for the quantitation of LP-X subsequent to electrophoresis. It is possible to excise the agar gel corresponding to the migration area of LP-X and quantitate LP-X by performing lipid phosphorus determinations (*17*). Alternatively, LP-X and other LDL fractions can be precipitated by polyanionic compounds, such as heparin, and metal salts, such as magnesium chloride, and the precipitated band of LP-X quantitated by densitometric scanning (*18*). Prior to electrophoresis on agar gel, if the serum is incubated with radiolabeled cholesterol, the label will equilibrate with the various lipoprotein fractions. Subsequent electrophoresis and determination of percent of radioactivity associated with the cathodal area coincident with the migration of LP-X is used to quantitate LP-X (*19*). The sensitivity of these modified electrophoretic procedures vary.

Electrophoresis, with subsequent precipitation by heparin and magnesium chloride, and densitometric scanning can quantitate LP-X in the range of 60 mg–6.3 g/liter. Levels above 6.3 g/liter, seen in cases of severe obstruction, can be quantitated upon appropriate dilution with sera free from LP-X. It must be noted that LP-X levels lower than 60 mg/liter can be encountered in early stages of cholestasis, which might escape detection by the above procedures.

Procedures employing electrophoresis with subsequent lipid phosphorus determination on the excised agar-gel portion containing LP-X are unable to quantitate levels below 500 mg/liter.

B. Immunochemical Techniques

The availability of antisera to highly purified LP-X preparations permits one to measure LP-X. However, procedures using radial immunodiffusion are time-consuming. Since antigenic determinants on LP-X are shared by other lipoproteins, cross-reactivity is also a problem. This has been circumvented by prior incubation of serum with antilipoprotein-B to precipitate lipoprotein-B and subsequent measurement of the supernatant containing LP-X by either radial immunodiffusion or electroimmunodiffusion. The electroimmunodifussion procedure can be performed in 3 hr in contrast to the 72 hr required for the radial immunodiffusion procedure. The former procedure can be shortened by another 75 min by performing the precipitation step with antilipoprotein-B on the agarose gel itself (*20*).

The sensitivity of immunochemical procedures is inferior to that attained by electrophoresis followed by precipitation with polyanionic compounds and densitometry. The radial immunodiffusion procedure (*18*) is

unable to quantitate LP-X levels below 300 mg/liter. The electroimmunodiffusion procedure is only slightly better with the lower limit of detection around 200 mg/liter.

Immunochemical procedures utilizing prior precipitation with antilipoprotein-B may not completely eliminate the cross-reactivity problem because some VLDL subspecies and lipoprotein-C related to HDL are not reactive with antilipoprotein-B. However, the levels of these species in human sera unrecognizable by antilipoprotein-B are negligible, and thus do not present a serious limitation in the quantitation of LP-X.

One must also be wary of pitfalls associated with electrophoretic procedures that are related to the sample and the electrophoretic media. LP-X bands seen in fresh sera are known to disappear upon storage. Batch to batch variability of agar used for preparation of the gel may have an effect upon the migration characteristics of LP-X.

VI. LP-X AND LCAT ACTIVITY

The appearance of LP-X in the plasma of patients with cholestasis is known to depress plasma levels of the LCAT enzyme. This enzyme catalyzes the transfer of the acyl group in the 2-position of phosphatidyl choline to the hydroxyl group of cholesterol. LP-X levels exceeding 2.5 g/liter completely abolish the LCAT activity in plasma (*21*). Correction of biliary obstruction by surgery leads to a decrease in LP-X accompanied by an increase in plasma LCAT activity (*22*).

The rigid structure of LP-X could partly explain why it is a poor substrate for LCAT, although the presence of short-chain fatty acyl donors, such as myristate, associated with the phosphatidyl choline of LP-X, should facilitate acyl group transfer (*5,23*). The presence of bile salts on the surface of LP-X and its characteristic free cholesterol to phospholipid ratio have been implicated in the inhibition of LCAT activity (*21*).

VII. CLINICAL USEFULNESS

The rare familial deficiency of the enzyme LCAT is associated with increased plasma LP-X levels. Our current knowledge is that there are subtle differences between LP-X associated with LCAT deficiency and the species encountered in cholestasis. Thus the apolipoprotein-A-1 (APO-A_1) found in LP-X associated with LCAT deficiency is found only in the LP-X_2 and LP-X_3 fractions of cholestatic LP-X and is missing in the LP-X_1 fraction (*5*). Electron micrographs of LDL fractions obtained from

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