DIRECT DETERMINATION OF CHOLESTEROL LIPID DISTRIBUTIONS USING CIRCULAR DICHROISM

By

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PREFACE

The correlation between high cholesterol concentrations in human blood and the risk for coronary heart disease has prompted a national effort to identify and treat all American adults who are at high risk because of elevated cholesterol levels. This effort has been very successful in increasing the awareness of the public about the importance of monitoring cholesterol levels. For this awareness to be truly beneficial requires that precise and accurate cholesterol measurement methods be used to identify those individuals at risk. At present, the reliability of serum cholesterol measurements in the United States points to the need for more accurate testing methods. The need for more accurate methods for determining cholesterol especially in association with the various lipoproteins has led to the development of a new method in which cholesterol levels are determined using circular dichroism spectropolarimetry.

I would like to thank all the necessary and helpful people who instructed, guided and encouraged my academic and research progress. Many thanks to my research advisor, Dr. Neil Purdie, for his enthusiasm for the cholesterol project and his guidance and encouragement of my endeavors. I would like to thank my committee members, Dr. Horatio Mottola and Dr. Ziad El Rassi, for their instruction in the principles of analytical chemistry and their support and encouragement. I am grateful to the faculty of the chemistry department for their instruction, advise and support. I
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To my children, Brett and Danica Murphy, who have kept me supplied with love, hugs and laughter, I say a very special thank you. Thank you to my parents, Don and Carolyn Haley, for having confidence in me and loving me.

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CHAPTER I

INTRODUCTION

The national effort to educate the general public about the increased risks of coronary heart disease (CHD) and artherosclerosis due to increased accumulation of arterial plaque caused by elevated serum cholesterol levels has been very successful. The public is now encouraged to know their cholesterol levels and cautioned that an uncontrolled diet and lack of exercise, as well as other factors such as age, gender, heredity, tobacco use and alcohol consumption can increase the risk of CHD (1,2). This has led to the necessity for automated methods for cholesterol determinations to handle the immensity of the screening program required. Cholesterol screening is now being done everywhere from shopping malls, supermarkets and other high traffic public areas to the more traditional hospital and laboratory settings. The methods used differ in complexity from the simple dip-stick method where a color sensitive reaction is measured on a paper support to more sophisticated lipid profile tests which determine the distribution of cholesterol among the three solubilizing macromolecules (3). The dip-stick method is used as a preliminary qualitative test to determine the need for a fuller more quantitative measurement.

A report (4) was prepared by the Laboratory Standardization Panel (LSP) of the National Cholesterol Education Program (NCEP) at the conclusion of a recent study of the relationship of health risk factors with elevated serum cholesterol levels. This study correlated the risk with three ranges of total cholesterol (TC). An individual was considered to be at: low risk if the TC was in the desirable concentration range of less than 200mg/dL; marginal risk in the borderline high range from 200-239mg/dL; and high risk for concentrations of 240mg/dL and greater. Individuals are judged to belong to one or

another of these risk categories based on the results of a serum TC measurement, then the other risk factors (1,2) are added and evaluated as a basis for patient counselling. Previous criteria for evaluating an individual's relative risk involved using a TC to high-density lipoprotein cholesterol (HDL-C) ratio (1,5). Low relative risk was indicated by a TC to HDL-C ratio of less than 5 and implied a high level of HDL-C. The HDL-C was measured in a second independent test for this evaluation method.

The report prepared by LSP (4) also described serious inaccuracies in measurement of the concentration of TC present in human serum reference standards, made by many clinical laboratories in their determinations. A 1985 survey, by the College of American Pathologists (CAP), in which results from 5,004 participating laboratories were submitted was cited to point out these inaccuracies. The results from laboratories using an enzymatic method were found to have 47% of their values to differ more than ±5% of the Centers for Disease Control (CDC) Confirmatory Value even after the values greater than 3 standard deviations from the mean value were removed. Of the 47%, about 16% were equal to or greater than ±10% and 8% were equal to or greater than ±15% of the CDC value. This prompted the recommendation by the LSP that the coefficient of variation (CV) be improved to within ±3% for TC by 1992. Although the results of similar surveys by the CAP in 1986 and 1987 were much the same as in 1985, more recent surveys of certified laboratories show that much progress is being made towards meeting the recommendations of the LSP using the current clinical methods and instrumentation (6). Inaccuracies in the determination of the distribution of cholesterol among the various lipoproteins were not reported by the LSP, but future evaluation was indicated. Important correlations between HDL-C levels and CHD are recognized but universal measurement of HDL-C is not recommended at this time because the current technology lacks the necessary reliability and proficiency (5,7,8). Interlaboratory CV's as high as 38% were reported in a recent publication (7). A 1987 CAP survey in which over two thousand laboratories analyzed the same sample for HDL-C showed a more than 5% difference from the reference value was

reported by more than one third of these laboratories. The inaccuracies in the current methods of determining HDL-C indicate that they lack predictive clinical value.

In the serum, cholesterol is distributed in association with high-density lipoproteins (HDL), low-density lipoproteins (LDL) and with triglycerides as the very low-density lipoproteins (VLDL). Statistical evidence from several long term clinical tests indicates that a high proportion of HDL-C and a low proportion of LDL-C is associated with lower relative CHD risk (1,2). This justifies that high levels of LDL-C are to be avoided while HDL-C is considered to be beneficial in higher proportions (5). There has been no direct implication of VLDL-C in any risk determination but high triglyceride can be a serious health problem by itself. A typical lipid profile study consists of the direct measurement of total and HDL cholesterols and direct measurement of triglyceride. The triglyceride is divided by five and this value is taken as the VLDL cholesterol. The VLDL-C and HDL-C values are then subtracted from the value for total cholesterol to obtain the LDL-C value. This makes the LDL-C the least accurately known fraction due to the propagation of any errors in the measurement of the other three fractions. This makes it difficult to monitor clinical progress in LDL-C reduction therapy with accuracy.

The goal of this research project was to develop a method for cholesterol screening which would allow HDL-C, VLDL-C and especially LDL-C to be determined directly in a single experiment. The selectivity of CD detection is such that this goal is not unreasonable, if the method of detection is coupled with an appropriate color-inducing reaction. A critical part of the research was to select a chemical reaction that would introduce, into the cholesterol molecule, a chromophore which absorbs light at a suitable wavelength and is situated near a chiral center. Many reactions were investigated but only the reaction ultimately considered to best meet the requirements is discussed in this report.

It was also sought to reconfirm the presence of excessive experimental error in conventional methods of determining HDL-C and further point out the need for new methods of testing. To assess the validity of the newly developed procedure, comparisons

were made between cholesterol lipid distribution data from separate laboratories using different methods of determination.

CHAPTER II

HISTORICAL AND THEORETICAL BACKGROUND OF CIRCULAR DICHROISM

History

The first recorded observation of optical activity was made in 1811 by Arago, a French astronomer, with the use of quartz plates he was able to rotate the plane of polarization of plane-polarized light (9,10). In 1812, Biot demonstrated that polarized light could also be rotated by solutions of some organic compounds (10,11) showing that optical activity was not limited to crystalline substances. In independent observations , Biot and Fresnel noted that the angle of rotation of light by a substance was increased as the wavelength of the incident light striking the substance was decreased (9). This effect is known as optical rotatory dispersion (ORD). In 1846 Haidinger observed the unequal absorption of left and right circularly polarized light by amethyst quartz crystals (9), identifying the phenomena now known as circular dichroism (CD).

In 1848 Louis Pasteur provided the first insights into the physical basis for optical activity. He used the term dissymmetry to describe the mirror image like difference in hemihedral crystals of a tartrate (12). In 1860 he explained molecular dissymmetry and the dissymmetry caused by the structure of certain crystals because of the molecular structure(11). Pasteur was able to physically separate crystals from a racemic mixture of sodium ammonium tartrate into it's two enantiomers and make solutions from each of these. Each of these solutions was found to rotate an incident beam of polarized light to a certain angle but each rotated the light in a different direction. From his experiments

Pasteur concluded that there were two categories of molecules: those that are superimposable mirror images and those that are nonsuperimposable mirror images (13). He concluded that molecules which exhibit optical activity were of the nonsuperimposable type (10).

In 1874 Van't Hoff proposed the existence of assymmetric carbon atoms with a tetrahedral configuration (14). This necessitated the use of three dimensional formulae to adequately describe organic molecules.

To understand the physical nature and causes of optical activity, studies were made using chiroptical techniques. In 1896 Aime Cotton first discovered CD in solutions of copper and chromium tartrates and used these solutions to investigate both CD and ORD (9). His research led him to believe that the curves produced by CD were the result of differences in absorbance of right and left circularly polarized light.

Theory

As defined by the IUPAC (16) an optically active substance is one which exhibits different interactions with left and right circularly polarized light. One type of optically active media is the inherently dissymetrical molecule (9). This type of optically active molecule requires the presence of an assymmetric carbon atom with a chromophore in close proximity. The substances discussed in this study are of this type. This interaction with circularly polarized light is the basis for the chiroptical techniques of polarimetry, ORD and CD (17).

The optical phenomena of circular dichroism (CD) and optical rotation are related to those for ordinary absorption and dispersion (13). Circular dichroism is the term for the difference in absorption coefficients of an optically active medium for left and right circularly polarized light, while circular birefringence is the difference in the refractive indices of the medium for the circularly polarized components.

To understand the differences in chiroptical and ordinary spectroscopic methods, an explanation of the electromagnetic radiation used in them is necessary. The wave phenomenon of light is caused by transverse vibrations of the electric field vector, Figure 1. A magnetic field vector is perpendicular to the oscillating electric field vector but can be ignored during this discussion . The electric field vibration is perpendicular to the direction in which the light wave travels and occurs in an infinite number of planes . The unpolarized monochromatic light used for UV visible spectroscopy consists of different wavelengths vibrating in many different planes.

Figure 2.a is a schematic representation of unpolarized light. Figure 2.b depicts linearly polarized light which vibrates in only one direction. This is the type of light utilized in chiroptical techniques. Linearly polarized light can be considered as the vector sum of its left and right circularly polarized components, Figure 3. Over time, as the wave travels along a given axis the electric field vectors of the left and right circularly polarized light trace out left and right handed helices. If the vectors are not allowed to propagate in distance, but only in time, the circular figures depicted in Figure 2.c and d result.

The incident linearly polarized light used to investigate optically active absorbing samples can be described as having the two circular components in phase with amplitudes which are equal but opposite in sign, Figure 3. These two components travel through the medium with different speeds due to the circular birefringence, $\eta_L - \eta_R$, of the medium. In the resultant transmitted wave the two circular components are no longer in phase and have unequal angles from the incident plane of polarization ($\omega \neq \omega'$). The major axis of vibration is rotated from that of the incident light by an angle α which is termed the optical rotation, Figure 3.b. When linearly polarized light passes through a sample that is not optically active the field vectors remain in phase, the angles of each of the two vectors with the incident plane are equal ($\omega = \omega'$), and the transmitted beam is still linearly polarized in the same plane as the incident beam, Figure 3.a.

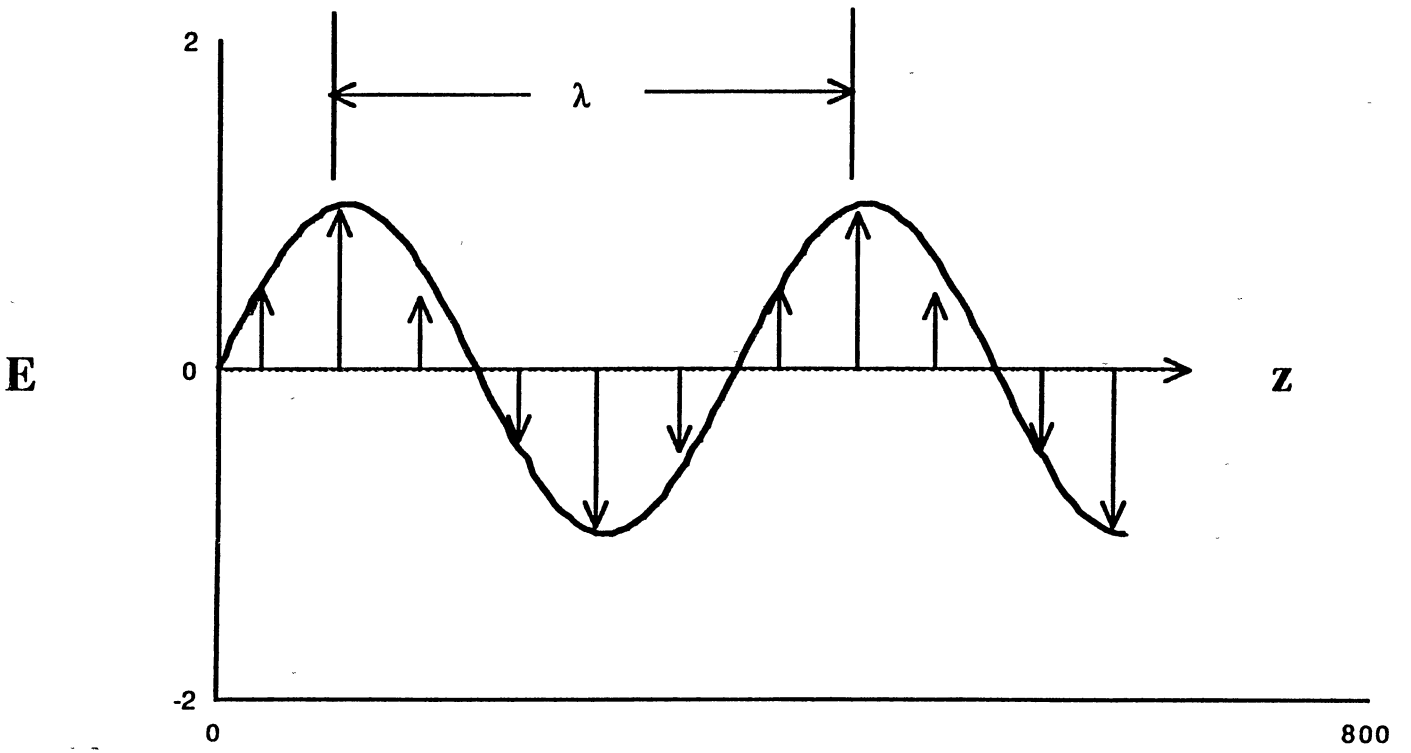


Figure 1. Transverse wave representation of the electric field associated with a monochromatic light beam. The arrows represent the magnitude of the oscillating electric field. The distance between cycles is related to wavelength, λ, of the radiation.

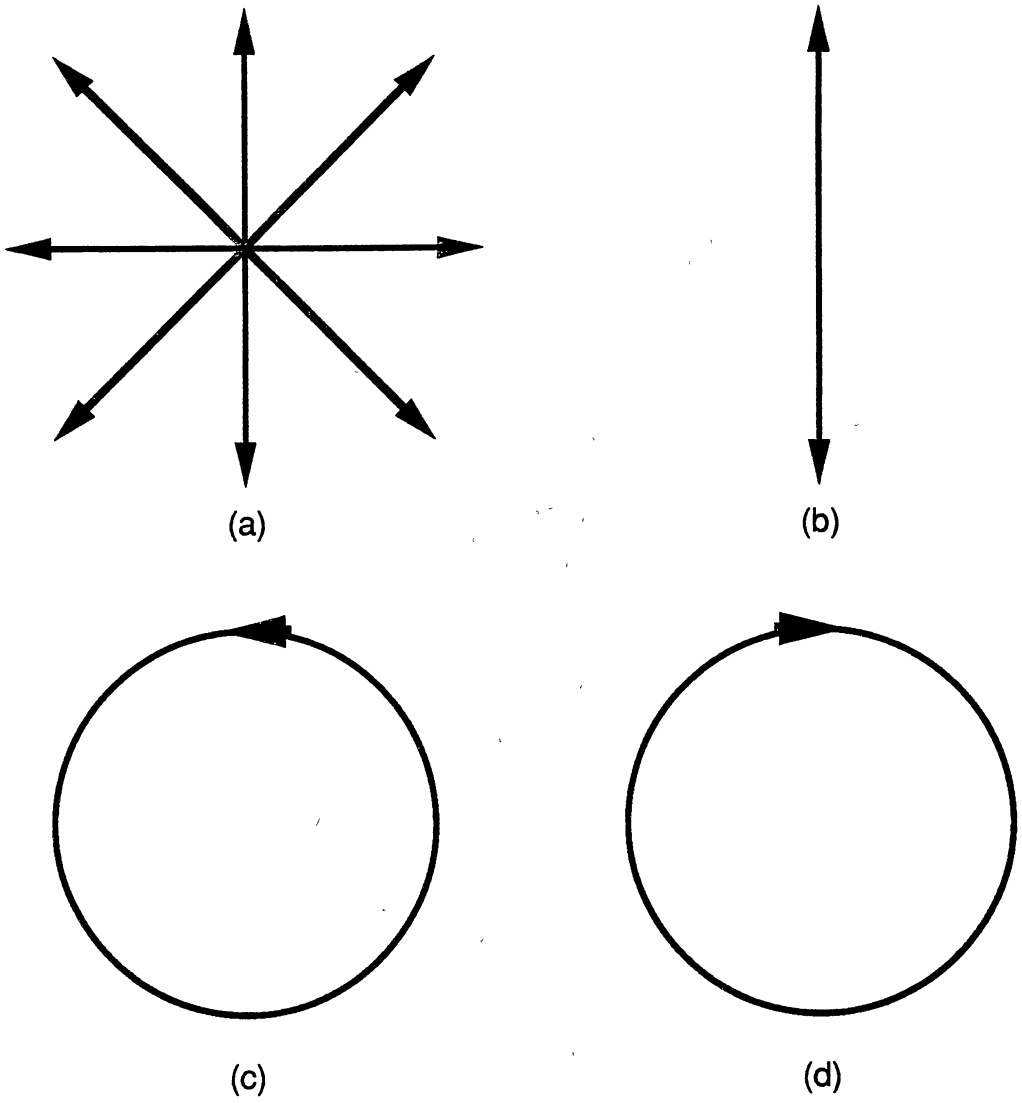


Figure 2. Polarization of monochromatic light beams: a) unpolarized light, b) linearly polarized light, c) left circularly polarized light, and d) right circularly polarized light. Direction of propagation is out of the page.

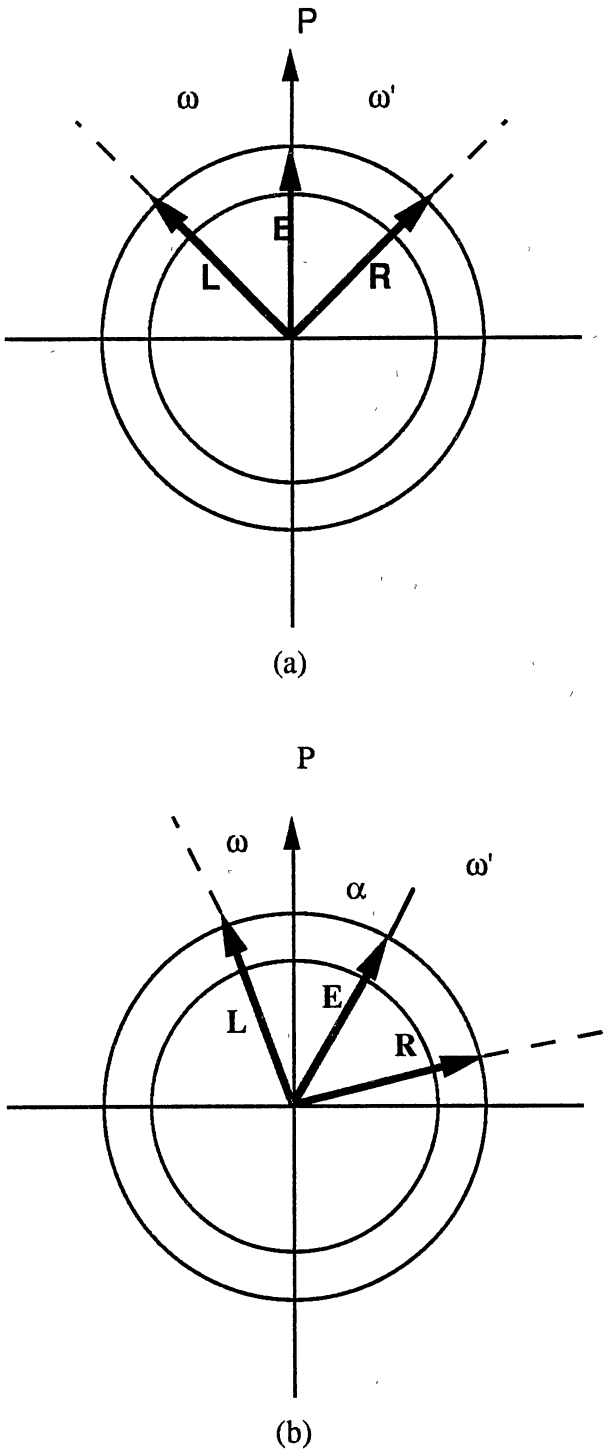


Figure 3. Direction of the electric field vectors emerging from a) an achiral medium and b) a chiral medium. P is the original plane of polarization, L and R are the left and right circularly polarized electric field vectors, E is the resultant electric field vector.

Fresnel correctly postulated that optical rotation in chiral subatances results from the difference in refractive index for the left and right circularly polarized light (9).

$$\eta L - \eta R \neq 0 \tag{1}$$

Differences in refractive indices account for differences in the velocities of the two circularly polarized components traversing the medium. The optical rotation, α, is directly proportional to the difference in refractive indices and is given by the equation

$$\alpha = 1800(\eta L - \eta R)/\lambda(cm) \tag{2}$$

and is expressed in degrees per decimeter.

To normalize the concentration, making it useful for comparison purposes, the quantity $[\alpha]$, the specific rotation, is introduced,

$$[\alpha] = \alpha/c'b \tag{3}$$

where c' is the concentration in g/cm^3 and b is the pathlength of the cell.

For comparison of solutions of different materials, the molar rotation, $[\phi]$, must be used,

$$[\phi] = [\alpha]M/100 \tag{4}$$

where M is the molecular weight in g/mole. Division by 100 keeps the numbers small (15) and has no physical meaning. This equation normalizes the optical rotation to molecular weight and facilitates comparison on a mole for mole basis.

As the incident linearly polarized beam passes through an achiral (optically active) medium there is also a difference in absorbance experienced by the two components.

$$\Delta A = A_L - A_R \neq 0 \tag{5}$$

The molar absorbance coefficient of a substance is related to the absorbance (A) by the Beer-Lambert law:

$$A = \varepsilon bc \tag{6}$$

where c is the concentration in moles/Liter, b is the pathlength of the cell and ε has units of liters per mole centimeter. The left and right circularly polarized components have different molar absorption coefficients. The signed difference is defined by IUPAC (13) as circular dichroism (CD).

$$\Delta\varepsilon = \varepsilon_L - \varepsilon_R \neq 0 \tag{7}$$

Most CD instruments measure the differential absorbance, $\Delta A = A_L - A_R$, which is related to the difference in molar absorption coefficients described in equation 7, by equation 8, in which c is in moles/liter and b is the pathlength.

$$\Delta A = \Delta\varepsilon cb \tag{8}$$

As a result of the combined differences in the refractive indices and the absorption of the left and right components of the linearly polarized light, the components of the transmitted beam, as well as being out of phase, are of unequal magnitude. Because of the difference in amplitude of the electric field vectors, depicted as L and R in Figure 4, the

resultant vector, E, traces out an ellipse and no longer oscillates in a single plane. The difference in amplitude of the field vectors combined with the circular birefringence produces a transmitted beam which is elliptically polarized and rotated by an angle a from the original plane of polarization. Equation 7, however, is only nonzero in areas of an absorption band thus the CD is only measureable in these regions (17). Since not all absorption bands are associated with a chiral chromophore not all absorption bands exhibit CD activity. This allows for greater selectivity in CD.

The arctangent of the ratio of the minor axis of the ellipse, OA, to the major axis of the ellipse, OB, is termed the ellipticity, ψ, as shown in figure 4, and is a characterization of the eccentricity of the elliptically polarized light. The equation is as follows:

$$\tan \psi = OA/OB \tag{9}$$

Just as $(\eta_L - \eta_R)$ is small compared to the index of refraction, the difference, $(\varepsilon_L - \varepsilon_R)$, between absorption coefficients is small (9).
This allows ψ to be quantitatively approximated as

$$\psi = \pi (\varepsilon_L - \varepsilon_R)/\lambda \tag{10}$$

where λ is the wavelength of the incident radiation. This equation is analogous to the equation for the optical rotation since optical rotation is characteristic of circular birefringence as ellipticity is characteristic of CD.

The analogy between the two characteristics carries over and is demonstrated by the similarity between the expressions for specific ellipticity, $[\psi]$, and specific rotation $[\alpha]$. The specific rotation, described mathematically, is given as

$$[\psi] = \psi/c'b \tag{11}$$

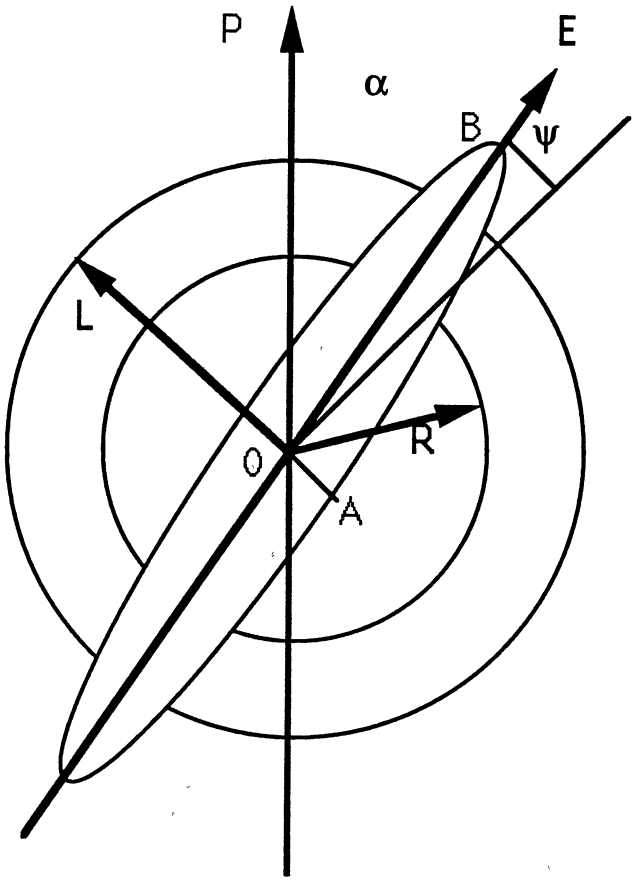


Figure 4. Production of elliptically polarized light in circular dichroism. P is the original direction of polarization, **L** and **R** are left and right circularly polarized light respectively, **E** is the resulting electric field which is now elliptically polarized.

where ψ is ellipticity measured in degrees, the concentration, c', is in g/ml and the cell path length, b, is measured in cm. This quantity is useful for comparing different concentration of the same substance.

For comparisons between different substances with different molecular weights, the molar ellipticity, $[\theta]$, provides a mole for mole basis. The molar ellipticity is analogous to the molar roatation and is defined as

$$[\theta] = [\psi]M/100 \tag{12}$$

with M being the molecular weight in g/mole. The analogous nature of these two equations, 4 and 12, direct comparison between the magnitude of the optical rotation and the ellipticity for an individual molecule is possible on a mole to mole basis. Also by analogy, the molar ellipticity is proportional to $\Delta\varepsilon$, the difference in the absorption coefficients:

$$[\theta] = 3300\Delta\varepsilon \tag{13}$$

where the numerical constant is the result of conversion factors and constants.

The term ellipticity is still used even though most of the CD spectropolarimeters available measure the absorbance difference instead of the ellipticity. Because it is a measure of the absorbance difference the Beer-Lambert Law applies and the measurement of CD data done in this lab utilizes a definition of the molar ellipticity, θ_M, that is similar to this law.

$$\theta_M = \Psi/cb \tag{14}$$

The ellipticity, ψ, is measured in degrees, c is the concentration in moles/L and b, the cell path length, is measured in cm. This choice of units leads to values much different than those reported in the literature, but more readily facilitate the quantification of data.

The spectra obtained from CD measurements differ distintly from those obtained using ORD. In a typical ORD spectrum for a substance without a chromophore the ORD curve will either rise or fall monotonically, with decreases in wavelength, corresponding to the change in the magnitude of the optical rotation. Since these curves usually contain no inflection points or changes in sign they are referred to as plain ORD curves, Figure 5.a. In molecules which possess an optically active chromophore the optical rotation increases rapidly as the absorption maximum wavelength of radiation is approached then, just before the absorbance maximum is reached, the magnitude decreases drastically, passing through zero rotation, until it reaches a minimum from which it increases at a slower rate. The resulting curve is a sigmoidal shaped curve rising out of a plain ORD curve as depicted in Figure 5.b. This type of curve is termed an anomalous ORD curve. Background rotation is one of the major drawbacks of ORD making it difficult to establish the baseline. Since $\Delta\varepsilon$ is significant only at wavelengths corresponding to an optically active absorption band background rotation is not a problem in CD. The CD spectrum may possess a shape similar to the corresponding absorption curve and maxima in both are often close. The anomalous peak and trough of the ORD curve corresponds to a single positive or negative maximum in the CD curve as shown in Figure 5.c. These two different characterizations are both named cotton effects after Aime Cotton, the French physicist who investigated the phenomena(9).

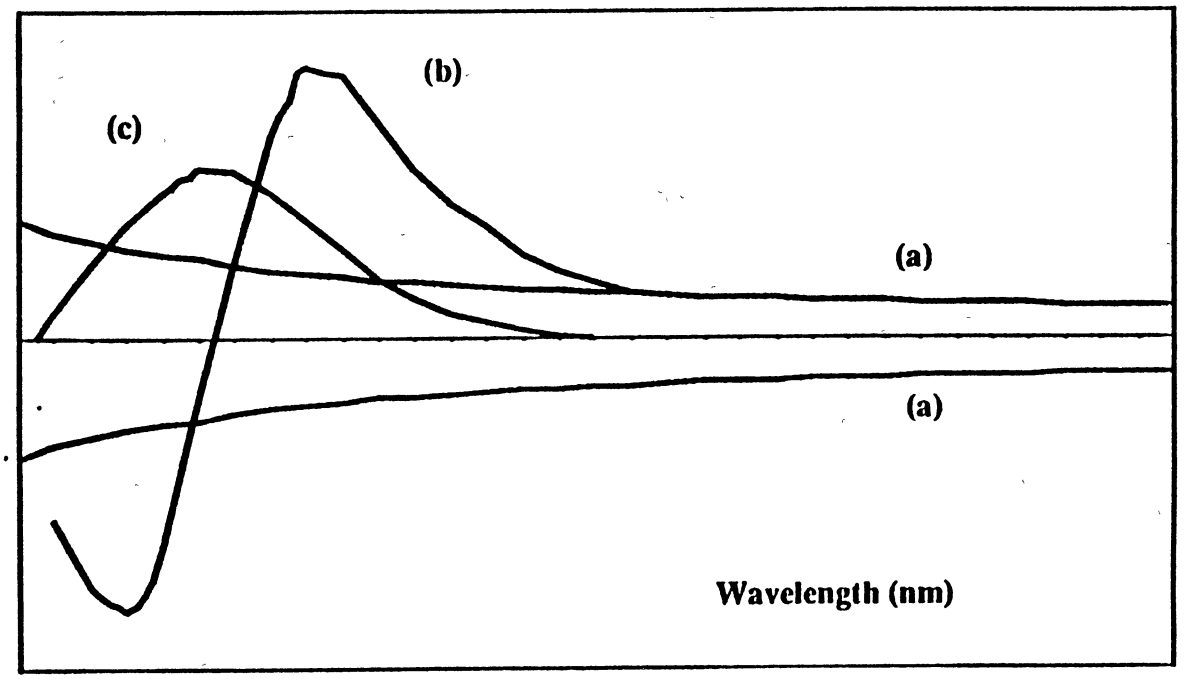


Figure 5. Typical chiroptical spectra: (a) plain ORD curves, (b) anomalous ORD curve with a single Cotton effect, and (c) CD curve with a single positive Cotton effect.

CHAPTER III

INSTRUMENTATATION

INTRODUCTION

All CD and UV spectra made in this laboratory were measured by a model J-500A automatic recording spectropolarimeter produced by Japan Spectroscopic Co., Ltd. (JASCO). A 450 watt xenon arc lamp is the light source for this instrument. The lamp is water cooled and is operated in a nitogen purged atmosphere so that ozone produced by the lamp does not damage the optical system. Initially the JASCO DP-500 data processor was used for data acquisition. CD spectra were recorded on a chart recorder and measurement of signal heights was done manually. Acquisition of a JASCO model IF-500-2 interface which allowed the J-500A to be coupled to an IBM-AT computer clone allowed the replacement of the data processor. The computer then measured CD signals digitally and the spectra were printed on a Hewlett-Packard 7475A graphics plotter.

The instrument was calibrated daily with a 0.025% (W/V) solution of androsterone in dioxane as suggested by JASCO (18). Instrument parameters such as the number of scans to be signal averaged and the sensitivity were adjusted to provide spectra quality in the minimum necessary time.

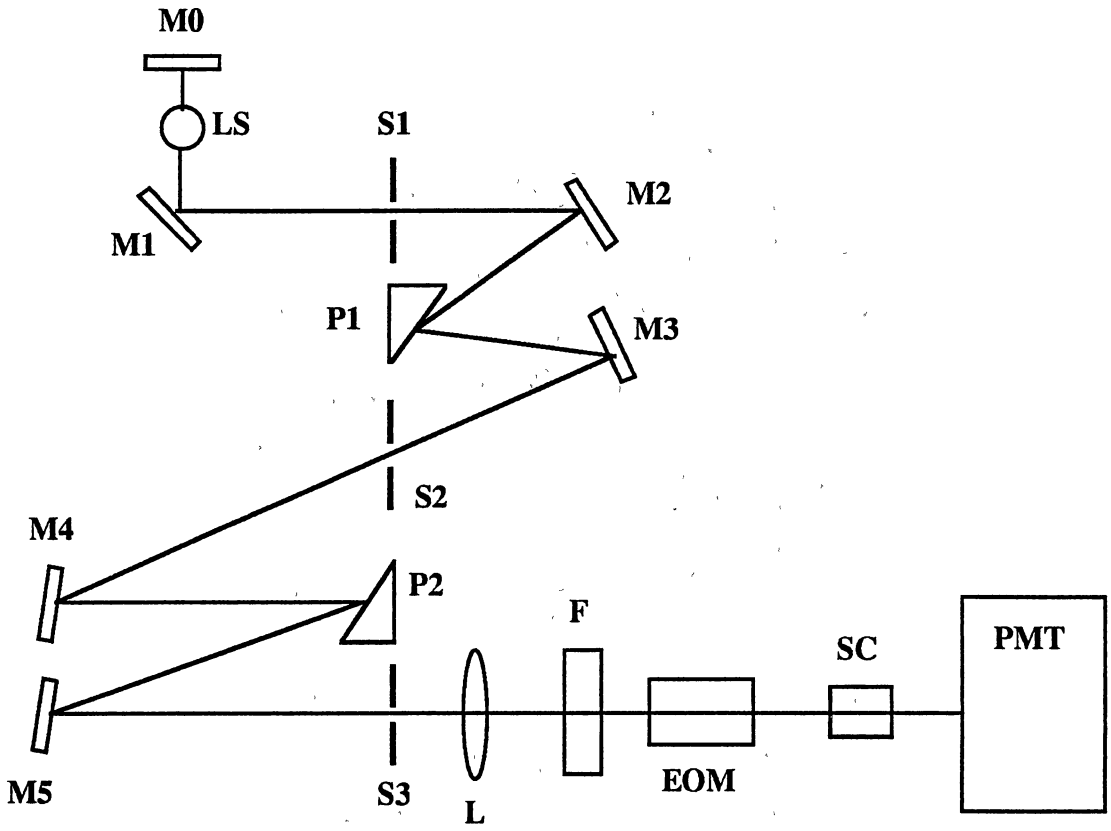
Description of the J-500A Optical System

CD spectropolarimetry requires a more complex optical system than conventional UV-visible spectrophotometry although the two systems are very similar. CD

measurements require monochromatic circularly polarized light in contrast to the simple monochromatic light necessary for ordinary spectrophotometric measurements.

A schematic representation of the J-500A optical system is shown in Figure 6. The light beam is focused on the entrance slit, S1, by a spherical mirror, M1. A double monochromator is an essential component of the J500A design because of the importance of keeping stray radiation to a minimum. S1 marks the entrance to the first monchrometer while the entrance to the second is marked by S2.

Prisms P1 and P2 are made of crystal quartz and their axial directions differ in respect to each other. This design allows them to serve the dual purposes of dispersion elements and birefringence polarizers. The light beam emerging from P2 is monochromatic and linearly polarized. Lens L focuses this beam onto filter F to filter any remaining unpolarized light. The circularly polarized light is produced by the electro-optic modulator designated as EOM. The J-500A utilizes a Pockels cell as the EOM. When an electric field is applied to the Pockels cell crystal a change in the refractive index and propagation velocity for the ordinary and extaordinary beams. These beams while linearly polarized and perpendicular to each other are out of phase. A phase difference of a quarter wavelength (π /2), or any uneven number of quarter wavelengths, produces a circularly polarized light beam. The Pockels cell alternately produces left and right circularly polarized light by changing the direction of the electric field which changes the direction of polarization. This is done at a frequency of 50 kHz. Once the light beams have traversed the sample cell interacting with the sample ,the photomultiplier receives and electronically recombines them.



M0, M1, M2, M3, M4, M5: spherical mirrors
LS: light source
S1, S2, S3: slits
P1: first prism (horizontal axis)
P2: second prism (vertical axis)
L: lens
F: filter
EOM: electro-optical modulator (Pockels cell)
SC: sample cell
PMT: photomultiplier tube

Figure 6. Optical system for the J-500A spectropolarimeter (adapted from reference 17).

CHAPTER IV

EXPERIMENTAL

INDEPENDENT LABORATORY METHODS

For this study cholesterol concentration determinations were made using three separate methods. Independent laboratories conducting broad range cholesterol screening programs provided the data for two conventional commercial methods. The Abbott Vision© clinical autoanalyzer, which will be designated as (A), was used by one laboratory. The DuPont aca© autoanalyzer, designated as (D), was used by the other laboratory. HDL-C concentrations were measured. The Trinder reaction, a double enzymatic multistep reaction process which produces a red form of a quinoneimine dye (18), is the basis of both commercial processes used. The enzymatic reactions involved in this process are as follows:

$$\text{Cholesterol Esters} + H_2O \xrightarrow[\text{Esterase}]{\text{Cholesterol}} \text{Cholesterol} + \text{Fatty Acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow[\text{Oxidase}]{\text{Cholesterol}} \text{Cholest-4-en-3-one} + H_2O_2$$

$$2H_2O_2 + \text{4-Aminoantipyrine} + \text{p-Hydoxybenzenesulfonate} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine Dye} + 4H_2O$$

UV-visible absorbance is the method of detection for these determinations. The absorbance intensity of the colored end product is proportional to the amount of cholesterol in the sample, although the quinoneimine dye is not structurally related to cholesterol. For the HDL-C determinations the same processes were used after the low density lipoprotein (LDL) and very low density lipoprotein (VLDL) cholesterol fractions had undergone a selective precipitation reaction with a prepared aliquot of dextran sulfate-Mg. Absorbance measurements at a single wavelength were made for 538 samples in the (A) data set and 130 samples in the (D) group.The data from these laboratories were collected over extended periods of time, up to two years for the laboratory using method (A). Triglyceride levels were measured for only 270 samples, but the values from these can be considered representative of the whole. TC and HDL-C were measured only once by these laboratories before they released the serum samples to our laboratory. The serum layer was removed and stored at 0-5°C upon receipt of the sample.

CD Detection Method

The experimental procedure used to obtain the third set of data was developed in the laboratory of Dr. Neil Purdie in the chemistry department of Oklahoma State University, and is the basis of a patent application filed with the U S Patent Office, January 1990. The reaction is totally a nonenzymatic chromogenic reaction attributed to Chugaev (19) and the method of detection is full spectrum circular dichroism (CD) spectropolarimetry (20), as opposed to simple absorbance. The reagent is a two to one mixture of 20% w/w anhydrous $ZnCl_2$ in glacial acetic acid and 98% acetyl chloride, as described by Chugaev. The structure of the colored end product is not certain although a mechanism in which the B-ring of the steroid nucleus is believed to open to produce an analog of Vitamin D has been suggested.

Only those compounds which exhibit optical activity and absorb electromagnetic radiation are detected by CD. Cholesterol and its esters meet these requirements but have a spectral maximum at 200nm. To simplify the measurement a color induction reaction is necessary for CD detection as it is with absorption detection. The reaction chosen must also produce a colored end product which meets the requirements of a chiral carbon with a chromophore in close proximity to facilitate CD detection. The Chugaev reaction produces such an end product. The Trinder reaction could not be used with CD detection since the colored quinoneimine end product is not optically active and therefore not detected.

CD detectors measure the difference between the absorbances, of the two circularly polarized components of linearly polarized light, as a function of wavelength (21). CD detection is more selective than absorbance and only CD-active compounds are potential interferences. The Chugaev reagent reacts with other steroids but because serum levels are too low and each steroid has a unique CD spectrum they do not interfere (22). The CD spectrum is not affected by turbid specimens in the same way as an absorbance spectrum since scattering of the coincident beams cancels when left and right absorbances are subtracted.

A step by step procedure was followed for each serum sample undergoing the Chugaev reaction. A 50mL aliquot of serum was placed in a 10mL vial and 2mL of the zinc reagent were added. Then 1mL of acetyl chloride is carefully added to the mixture which is then capped and thoroughly shaken. The solution is incubated at 67° for 8 minutes during which time a reddish-orange color develops. The mixture is cooled and 1mL of chloroform is added. It is then transferred to microcentrifuge tubes, centrifuged for 2 minutes and transferred to a quartz spectrophotometric cuvet. The CD spectrum is then measured from 625-325nm.

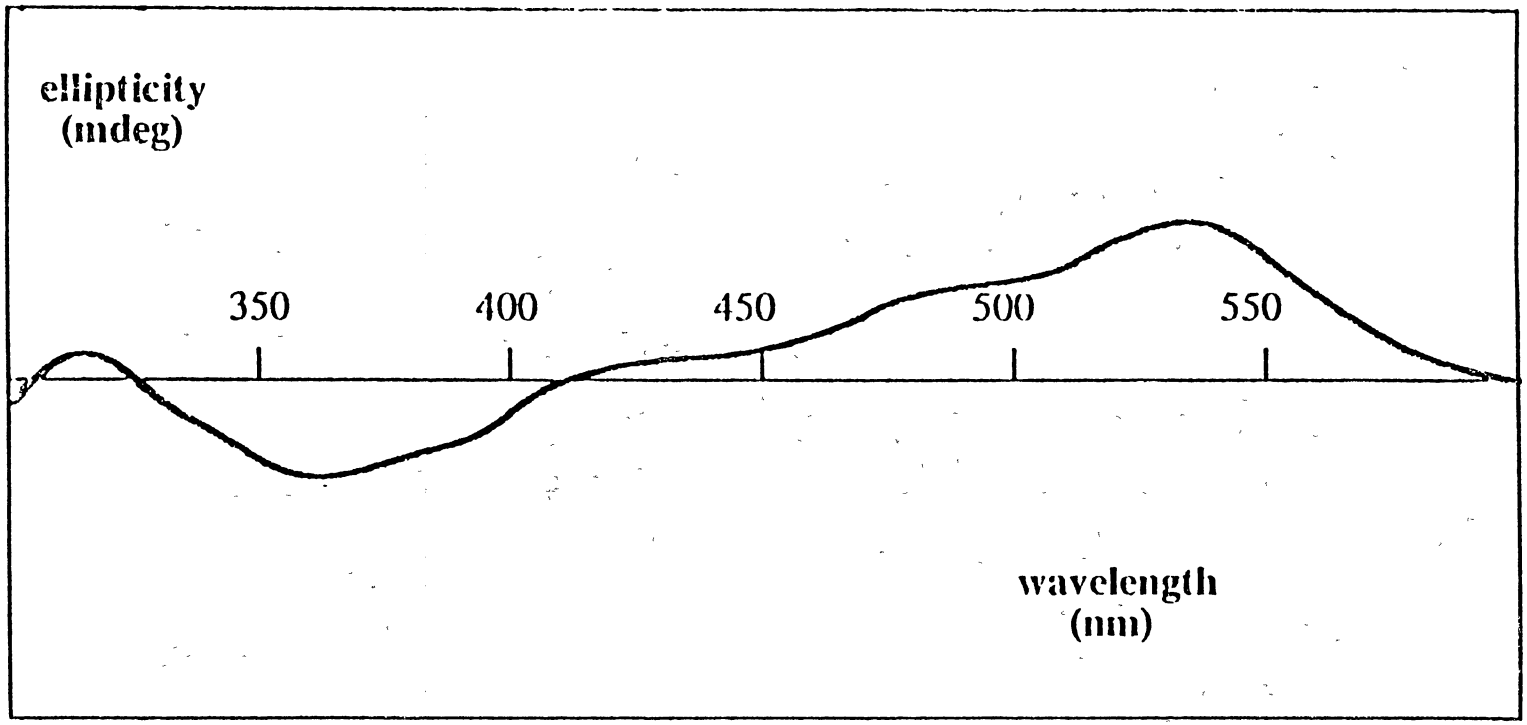


Figure 7. CD spectrum of the colored end product of the color reaction between the Chugaev reagent and an NMS cholesterol standard reference material.

The data set that was measured using the Chugaev-CD procedure included 134 serum samples. This data set consisted of sets of samples taken at random from the serum samples provided by laboratories (A) and (D). Triglyceride data were not available for all of the 134 samples.

Standard Materials

The National Bureau of Standards (NBS) cholesterol standard reference material (SRM911a) in chloroform was reacted with the Chugaev reagent and the colored end product produced the CD spectrum shown in Figure 7. Determinations on a series of dilutions of the standard were made and the data obtained were used to prepare calibration curves at a number of wavelengths. Analogous spectra were obtained for reactions with serum cholesterol and with standard solutions of cholesterol fatty acid esters in chloroform, which suggests that the cholesterol is totally converted to the the acetate ester under Chugaev reaction conditions.

Standard Reference Materials for Cholesterol in Human Serum (Frozen) were obtained from NBS at three different concentrations as listed in Table 1. The NBS literature stated that the samples were donated by the Center for Disease Control (CDC) and that the TC measurements were made at NBS with nine separate vials of each being measured twice each. The Certificate of Analysis accompanying these standards reported that the NBS data for each compared very well with the CDC measurements which used a modified Abell-Kendall method. Ten Determinations were made on each reference standard using the Chugaev-CD procedure and the data obtained (shown in Table 1) indicates that this procedure is valid for measurement of total cholesterol.

TABLE 1

COMPARISON OF DATA ON NBS
STANDARD REFERENCE MATERIALS

REFERENCE NUMBER	NBS CHOLESTEROL MEASUREMENT	CD CHOLESTEROL MEASUREMENT
SRM(1951-1)	210.36±2.46mg/dL	206.O±3.76mg/dL
SRM(1951-2)	242.29±1.53mg/dL	241.1±2.89mg/dL
SRM(1951-3)	281.97±1.82mg/dL	286.0±2.62mg/dL

Confirmation of the band assignments was obtained by two methods. The precipitating reagent, phosphotungstate-Mg, was added to precipitate the VLDL-C and the LDL-C fractions, according to the Sigma 353-2 procedure, leaving the HDL-C fraction. The spectrum obtained after precipitation is shown in Figure 8(c). Selective separation of the α-lipoprotein fraction, which is associated with HDL-C, on a heparin-agarose stationary phase according to the procedure developed by ISOLAB® was performed as the second confirmation method and resulted in spectra similar to that depicted in Figure 8(c).

CHAPTER V

RESULTS AND DISCUSSION

As previously stated the data set for CD detection consists of 134 serum samples. The TC for each sample was determined and these data are presented in Table 2 along with the corresponding TC measurement from the independent laboratories (A) and (D). It is recognized that the absence of comparative measurements of the same sample from all three laboratories detracts from the completeness of the analysis but we were unable to do this due to a lack of funds. Since both independent laboratory methods utilized the Trinder reaction with absorption in the visible region as the detection method it is deemed valid to combine these data into one set in further analyses.

It is possible to directly measure the HDL-C and the combined (VLDL+LDL)-C fractions separately due to the selectivity of the CD detector. This can be accomplished in a single experiment which does not require a precipitation step. Figure 8 shows typical spectra for: (a) total serum cholesterol in all its forms; (b) the (VLDL+LDL)-C fraction, this spectrum was obtained by subtracting the spectrum for HDL-C from the TC spectrum; and (c) the HDL-C fraction which was measured after the previously described precipitation reaction. From these spectra it can be seen that measurement at 525nm leads to direct detection of the (VLDL+LDL)-C fraction since the HDL-C fraction does not contribute to the band at this point. The HDL-C fraction is determined by measurement at either 475nm or 390nm or both. It is considered that the most precise numbers for HDL-C result when the calibration is based on the difference between the two, which avoids problems associated with any sample to sample drift in the baseline that may occur. The values for

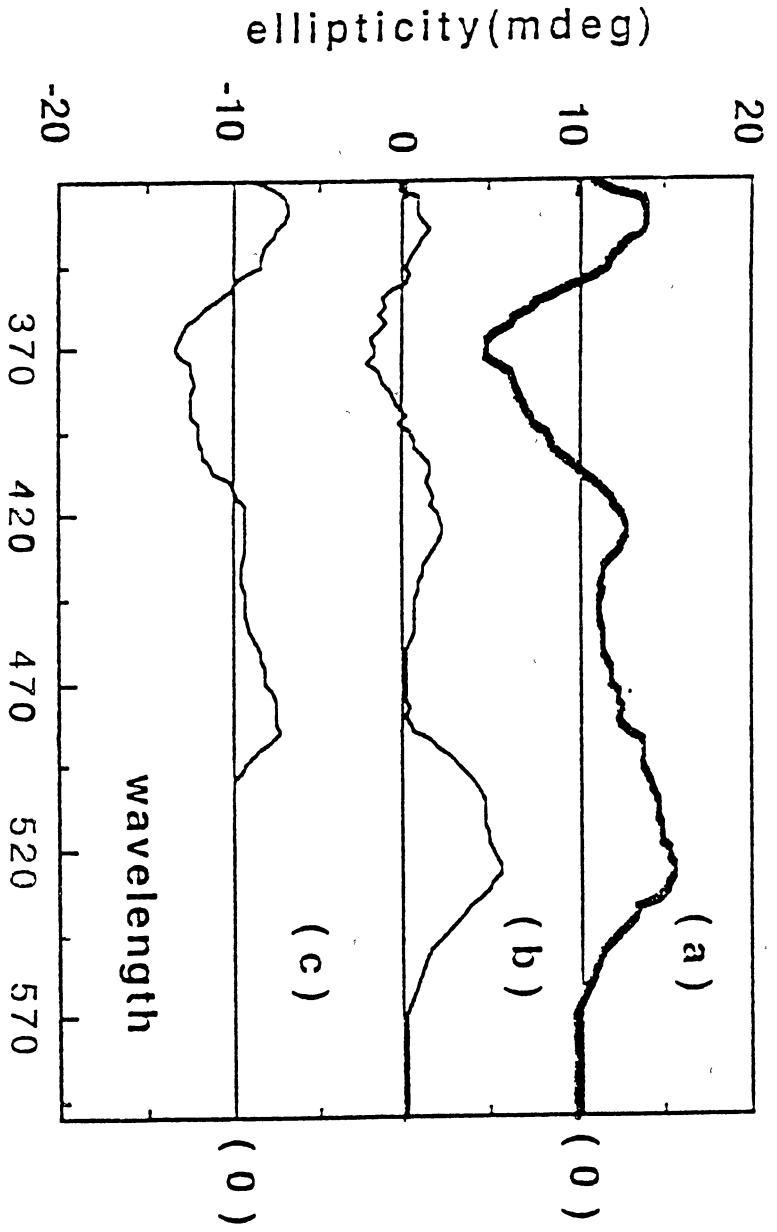


Figure 8. CD spectra for (a) total serum cholesterol; (b) the (VLDL+LDL)-C fraction, equal to (a) minus (c); (c) the HDL-C fraction after the addition of phosphotungstate-Mg precipitating agent.

TABLE 2

TOTAL CHOLESTEROL DATA FROM
(A), (D) AND CD LABORATORIES

Patient	Laboratory (A)	Laboratory (D)	CD Laboratory
LHM/001	258.0		271.9
LHM/002	264.0		290.0
LHM/003	229.0		185.8
LHM/004	221.0		212.3
LHM/005	211.0		225.1
LHM/006	281.0		290.9
LHM/007		203.7	195.2
LHM/008		219.6	204.2
LHM/009		159.5	157.3
LHM/010	255.0		259.8
LHM/011	293.0		278.4
LHM/012	203.0		214.3
LHM/013	185.0		170.7
LHM/014	168.0		167.3
LHM/015		231.2	226.9
LHM/016		233.0	239.5
LHM/017		309.1	264.4
LHM/018	294.0		268.0
LHM/019	220.0		237.1
LHM/020	227.0		256.1
LHM/021	192.0		197.4
LHM/022	134.0		146.2
LHM/023	166.0		172.4
LHM/024	166.0		182.6
LHM/025	254.0		273.1
LHM/026	245.0		244.9
LHM/027	179.0		177.9
LHM/028	188.0		183.5
LHM/029	188.0		208.9
LHM/030	214.0		219.4
LHM/031	219.0		218.3
LHM/032	194.0		198.8
LHM/033	127.0		132.4
LHM/034	291.0		335.0
LHM/035	252.0		232.9
LHM/036	163.0		184.2
LHM/037	189.0		222.2
LHM/038	232.0		243.5
LHM/039	192.0		193.5
LHM/040	114.0		138.0
LHM/041	96.0		114.0
LHM/042	208.0		231.0
LHM/043	200.0		196.0
LHM/044	249.0		227.0
LHM/045	197.0		198.0

TABLE 2 (Continued)

Patient	Laboratory (A)	Laboratory (D)	CD Laboratory
LHM/046	313.0	336.5	323.2
LHM/047	153.0	154.5	191.3
LHM/048	177.0	186.3	219.7
LHM/049	192.0	213.8	234.5
LHM/050	258.0	251.5	283.7
LHM/051	290.0		320.3
LHM/052	273.0		314.3
LHM/053	229.0		262.0
LHM/054		223.9	234.9
LHM/055		238.3	249.3
LHM/056		189.6	195.2
LHM/057		306.2	286.4
LHM/058		314.7	325.0
LHM/059		305.7	300.9
LHM/060		260.4	254.5
LHM/061		319.2	321.4
LHM/062		276.1	263.0
LHM/063		245.0	279.0
LHM/064		287.5	321.3
LHM/065		291.8	332.1
LHM/066		252.0	278.7
LHM/067		254.9	253.5
LHM/068		386.5	369.5
LHM/069		274.3	301.6
LHM/070		257.0	275.5
LHM/071		268.4	289.0
LHM/072		159.7	171.7
LHM/073	149.0	151.5	170.5
LHM/074	101.0	112.4	173.1
LHM/075	186.0	205.2	220.0
LHM/076	144.0	151.0	177.0
LHM/077	117.0	124.1	160.4
LHM/078	223.0	221.9	295.4
LHM/079	202.0	212.3	239.8
LHM/080	183.0		200.2
LHM/081	205.0		264.5
LHM/082	213.0		248.3
LHM/083	245.0		257.0
LHM/084	219.0		239.3
LHM/085		257.3	251.7
LHM/086		265.3	296.5
LHM/087		242.2	233.5
LHM/088		270.5	255.2

TABLE 2 (Continued)

Patient	Laboratory (A)	Laboratory (D)	CD Laboratory
LHM/089		292.7	301.9
LHM/090		245.7	258.9
LHM/091		252.6	267.5
LHM/092		242.3	284.2
LHM/093		225.0	248.0
LHM/094		199.4	215.0
LHM/095		246.7	260.5
LHM/096		185.5	210.6
LHM/097		230.9	209.4
LHM/098		274.5	279.5
LHM/099		293.1	278.0
LHM/100		256.8	237.6
LHM/101		256.0	251.8
LHM/102		251.7	257.9
LHM/103			285.6
LHM/104		265.3	258.5
LHM/105		196.6	203.7
LHM/106		345.9	342.6
LHM/107		271.3	271.1
LHM/108		294.5	308.8
LHM/109		282.5	300.1
LHM/110		222.7	212.7
LHM/111		226.1	208.4
LHM/112		234.9	233.2
LHM/113		293.1	274.2
LHM/114		267.4	275.7
LHM/115		273.9	293.0
LHM/116		308.6	317.8
LHM/117		264.8	261.3
LHM/118		231.2	215.0
LHM/119		263.5	252.7
LHM/120		219.7	218.2
LHM/121		211.3	229.0
LHM/122		201.9	219.0
LHM/123		234.3	280.6
LHM/124		183.8	196.2
LHM/125			290.6
LHM/126			326.8
LHM/127			307.7
LHM/128			271.5
LHM/129			318.6
LHM/130			334.0
LHM/131			337.7
LHM/132			366.0
LHM/133			268.1
LHM/134			252.1

these subfractions are combined to give the TC value. It is not possible to discriminate between the VLDL-C and LDL-C fractions at this time.

Table 3 presents the data from the measurement of distribution between the various lipid fractions made using the Chugaev-CD reaction (CD) and the combined results of the enzymatic methods (A)(D). The VLDL data are those provided by the independent laboratories and were calculated as 20% of the triglyceride. This data set is composed of 99 serum samples.

The attractiveness of enzymatic processes for serum cholesterol measurement is due to the selectivity of enzymes as reagents. The detection method of absorbance, however, is not selective and has many potential sources of interference. In comparison the chromogenic Chugaev-CD procedure utilizes a reagent selective for steroids and full spectrum CD detection which is selective enough to discriminate not only among these steroids, but also between the high density and combined low density lipoprotein cholesterol subfractions as well. There are apparently no interferences with this detection method.

No evidence was found that suggested that the choice of heparin or EDTA as the anticoagulating agent, by either independent laboratory, affected the results from CD detection. Within the Chugaev-CD data set the observed coefficient of variation for (VLDL+LDL)-C was ±2.3%. For HDL-C the imprecision was calculated using 390 and 475nm difference data and was found to be ±6.3% CV. The ±2.3% CV observed for the (VLDL+LDL)-C fraction is well within the recommended range for TC of ±3% CV that was proposed by the LSP for 1992. An improvement over the figures quoted in the introduction is shown using the CD method for imprecisions in HDL-C measurements. The LSP February 1990 (23) report figures include data from laboratories using both the (A) and (D) procedures and these figures are taken to be typical of what might be expected from the two independent laboratories contributing to this study.

TABLE 3

TOTAL CHOLESTEROL AND LIPID PROFILES
FROM CD AND (A)+(D) LABORATORIES

Patient	TC-(CD)	LDL-(CD)	HDL-(CD)	VLDL-(A)(D)	TC-(A)(D)	LDL-(A)(D)	HDL-(A)(D)
SS001	273	174	57	43	254	148	63
SS002	245	176	36	33	245	159	53
SS003	191	134	40	17	179	117	45
SS004	184	127	24	33	188	102	53
SS005	209	113	74	22	188		
SS006	219	84	47	88	214		
SS007	178	126	26	25	214	146	48
SS008	199	137	38	24	194		
SS009	132	97	22	14	127		
SS010	197	133	42	22	192	129	41
SS011	172	127	24	22	166	104	40
SS012	268	162	61	45	294	193	56
SS013	183	127	39	17	166	86	63
SS014	410		66				
SS015	260	176	64	20	255	176	59
SS016	278	167	72	40	293	198	55
SS017	214	139	52	23	203	140	40
SS018	171	86	58	27	185	115	43
SS019	167	103	44	20	168	94	54
SS020	186	114	44	27	229	140	62
SS021	212	142	42	28	221	140	53
SS022	225	170	28	27	211	139	45
SS023	291	184	39	68	281	183	30
SS024	272	194	57	21	258	194	43
SS025	290	213	57	21	264	200	43
SS026	101	65	17	19	106		
SS027	221	160	39	22	206		
SS028	205	156	31	17	182		
SS029	256	171	34	52	229		
SS030	335	196	47	92	291		
SS031	233	155	47	31	252		
SS032	184	144	26	15	163		
SS033	222	122	38	62	189		
SS034	244	160	40	43	232		
SS035	194	148	31	14	192		
SS036	138	100	27	11	114		
SS037	114	76	26	11	96		
SS038	231	136	61	34	208		
SS039	196	152	26	18	200		
SS040	227	181	26	21	249		
SS041	197	140	40	16	197		
SS042	235	189	28	18	224	144	62
SS043	249	180	38	32	238	171	35
SS044	195	152	30	13	190	136	40
SS045	286	250	21	16	306	233	57
SS046	325	237	52	36	315	229	50
SS047	301	227	50	24	306	230	52
SS048	254	198	37	20	260	181	60

TABLE 3 (Continued)

Patient	TC-(CD)	LDL-(CD)	HDL-(CD)	VLDL-(A)(D)	TC-(A)(D)	LDL-(A)(D)	HDL-(A)(D)
SS049	321		43		319		44
SS050	263	183	45	35	276	187	54
SS051	279	198	61	20	245	166	59
SS052	312	200	69	43	288	206	38
SS053	332	212	57	63	292	195	34
SS054	279	210	46	22	252	165	65
SS055	254	207	36	11	255	190	99
SS056	370	244	63	62	386	291	33
SS057	302	207	56	39	274	203	33
SS058	276	203	28	44	257	169	44
SS059	289	197	37	55	268	183	31
SS060	172	100	30	48	160	115	36
SS061	252	139	50	63	257	149	46
SS062	296	141	64	91	265		
SS063	234	163	45	25	242	163	54
SS064	255	130	58	62	270	167	41
SS065	302	200	71	31	293	221	41
SS066	259	182	42	34	246	176	36
SS067	268	207	42	18	253	182	53
SS068	284	218	44	22	242	173	48
SS069	248	170	28	50	225	127	48
SS070	214	146	43	25	199	131	43
SS071	260	177	42	42	247	162	43
SS072	211	161	34	15	186	134	37
SS073	209	163	34	13	231	155	63
SS074	280	209	40	31	274	214	30
SS075	278	215	43	20	293	214	59
SS076	238	178	37	23	257	196	38
SS077	252	173	42	37	256	172	47
SS078	258	190	50	28	252	178	46
SS079	286		27				
SS080	258	200	24	35	265	191	39
SS081	204	152	38	14	197	117	66
SS082	343	225	87	31	346	252	63
SS083	271	187	51	33	271	190	48
SS084	309	233	57	19	294	199	77
SS085	300	172	57	71	282	174	37
SS086	213	159	40	13	223	145	65
SS087	208	146	42	20	226	135	71
SS088	233	166	46	21	235	172	42
SS089	276	195	59	22	267	191	55
SS090	293	214	42	37	274	202	35
SS091	318	248	47	23	309	216	69
SS092	261	179	46	36	265	140	89
SS093	215	167	26	22	231	153	56
SS094	253	195	44	14	264	174	76
SS095	218	143	31	45	220	133	42
SS096	229	156	46	27	211	142	42
SS097	220	155	45	20	202	149	33
SS098	281	177	53	51	234	126	58
SS099	196	141	42	13	184	126	45

The excellent precision with which the direct measurement of the combined low density lipid cholesterol level is made is considered to be the most significant result of this research. For purposes of monitoring LDL-C levels in reduction therapy, it seems unfortunate that information on the LDL-C fraction can not be separated from that for the VLDL-C fraction, yet the ISOLAB® procedure has the same problem since both fractions coelute from the heparin-agarose stationary phase combined as the b-lipoprotein fraction. The Chugaev-CD procedure introduced here is more precise in the measurement of HDL-C than the Trinder-absorption procedure and offers direct determination of the combined low density fraction, therefore it is considered superior to these conventional commercial methods.

Specific descriptions of the interrelationships that exist among the three data sets which comprise this study are the emphasis of the remainder of this section. Figure 9 shows excellent correlation between the TC data from the Trinder (measured values) and the Chugaev (calculated values) methods. The correlation slope is 0.918 and the y-intercept is -25.9mg/dL. Both the (A) and (D) procedures are clinically approved and both use the same reaction, therefore it is considered valid to combine both sets of TC data into one group. It is clearly evident that the Chugaev-CD method for the measurement of TC is valid.

Comparisons between the commercial enzymatic and Chugaev-CD methods are limited to HDL-C and (VLDL+LDL)-C vs. TC data since no new method to measure VLDL-C was found. Data for these subfractions are plotted as a function of TC in Figure 10 for (A), in Figure 11 for (D) and in Figure 12 for Chugaev-CD. Separate correlations for VLDL-C and LDL-C data are included in Figures 10 and 11. For the (A) and (D) data sets correlations are the same and, with the exception of HDL-C, all are linearly dependent on TC. All of the HDL-C data measured by the enzymatic processes can be fitted by the value range of 50±10mg/dL for the whole concentration range of TC. The imprecision in HDL-C measurements are propagated into the LDL-C values since they are calculated using

the HDL-C measurement. The non-zero intercept of -50mg/dL and the correlation slope of almost 1.0 for plots of (VLDL+LDL)-C vs. TC for both data sets make the carry over of error obvious. The plot of (VLDL+LDL)-C vs.TC becomes a plot of TC minus a constant vs. TC for all practical purposes, given that a common value of 50mg/dL can be used to fit all HDL-C data measured enzymatically. For LDL-C calculated separate from VLDL-C the intercept is again about 50mg/dL,when plotted against TC, with a slope reduced to around 0.85 due to the linear dependence of VLDL-C with TC. It appears that LDL-C values can be estimated by assuming a constant value for HDL-C as accurately as they can be calculated using measured HDL-C values.

Determinations made using the CD method gave excellent correlations for both (VLDL+LDL)-C and HDL-C as a funtion of TC, Figure 12. Slopes correspond with figures that, based upon ultracentrifugation data (24), are considered to be reasonable distributions of total cholesterol among the various lipid fractions. The figures showed (VLDL+LDL)-C to be approximately 85% of TC and HDL-C the remaining 15%. Using VLDL-C data from the TGL measurements to separate the low density fractions in the CD measurements resulted in a LDL-C vs. TC plot which is linear with a y-intercept of 4mg/dL and a (LDL-C)/TC slope of about 0.68, Figure 12. It seems logical that the excellent precision with which the combined low density fraction can be determined could allow this measurement to be applied to LDL-C reduction therapies especially considering that VLDL-C is only a small part of this measurement. A proposal was included in the LSP report (6) that defined risk categories in terms of LDL-C levels rather than on TC. Table 4 summarizes the risk categories.

TABLE 4

RISK CATEGORIES BASED ON LDL-C AND TC CONCENTRATIONS

Health Risk	LDL-C	TC
Low	<130 mg/dL	<200 mg/dL
Moderately High	130-159mg/dL	200-239mg/dL
High	>160 mg/dL	>240 mg/dL

The ranges are based on the assumption that LDL-C is about 66% of the total cholesterol, on the average, which is basically what is shown for the variation of LDL-C with TC using the CD procedure.

Considerable improvement in the precision of HDL-C determinations using the Trinder reaction are required if the LSP recommendations are to be met. A good alternative would be to directly measure the (VLDL+LDL)-C using the Chugaev-CD procedure and redefine the risk assessment ranges at 85% of the cut-off values for TC. This would eliminate any errors in the assumption that everyone, regardless of physical condition, has a VLDL-C concentration equal to 20% of their TGL level.

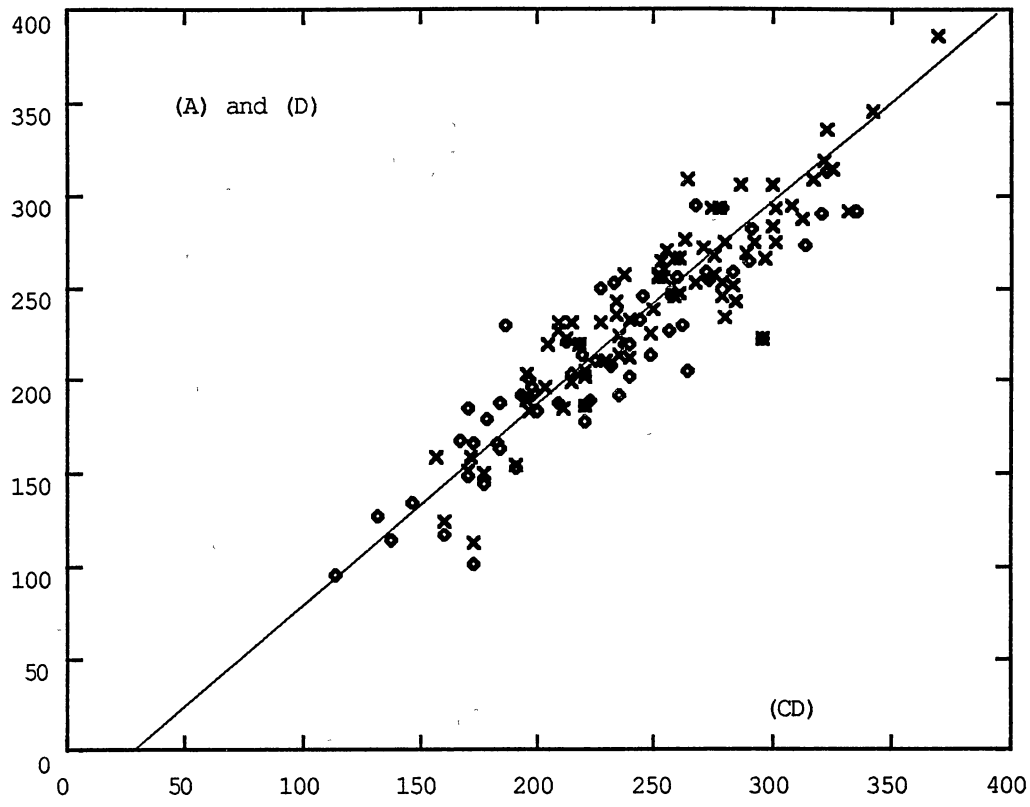


Figure 9. Total Cholesterol (CD) vs. Total Cholesterol (A) and (D). Least squares equation is y = -25.9 + 0.918x

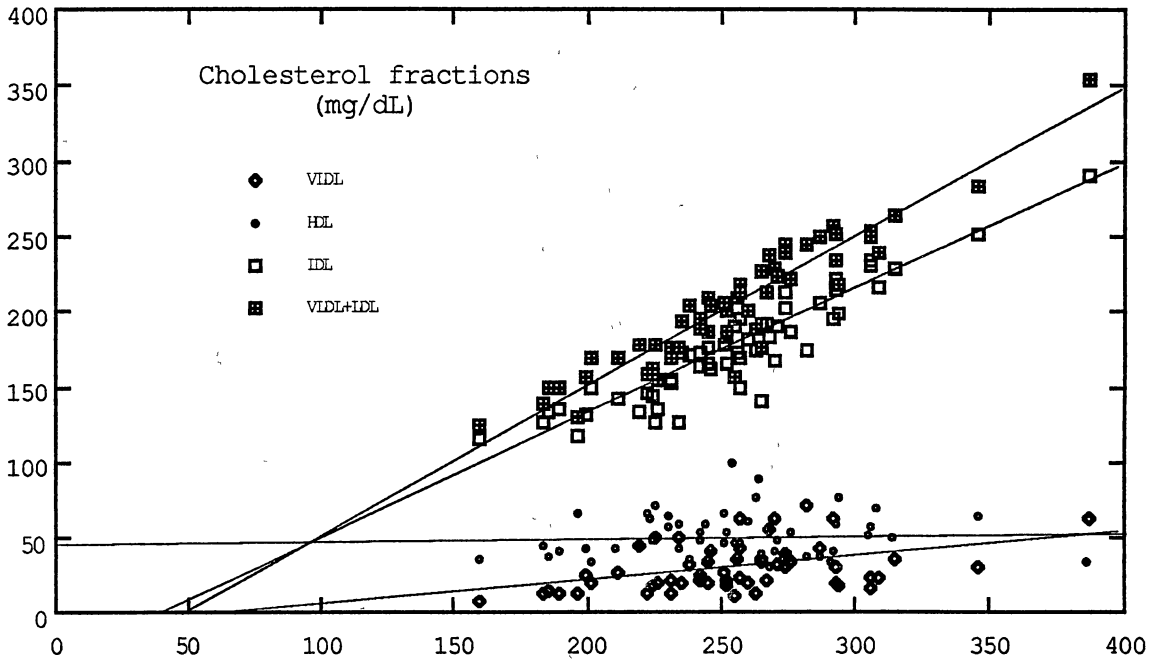


Figure 10. TC vs. HDL-C, VLDL-C, LDL-C, and (VLDL+LDL)-C for Laboratory (A). Correlation equations are:

(a) $y = 44.5 + 0.002x$ ($R^2 = 0.004$);

(b) $y = -10.5 + 0.16x$ ($R^2 = 0.180$);

(c) $y = -33.4 + 0.82\ x$ ($R^2 = 0.854$); and

(d) $y = -44.5 + 0.98x$ ($R^2 = 0.878$) respectively.

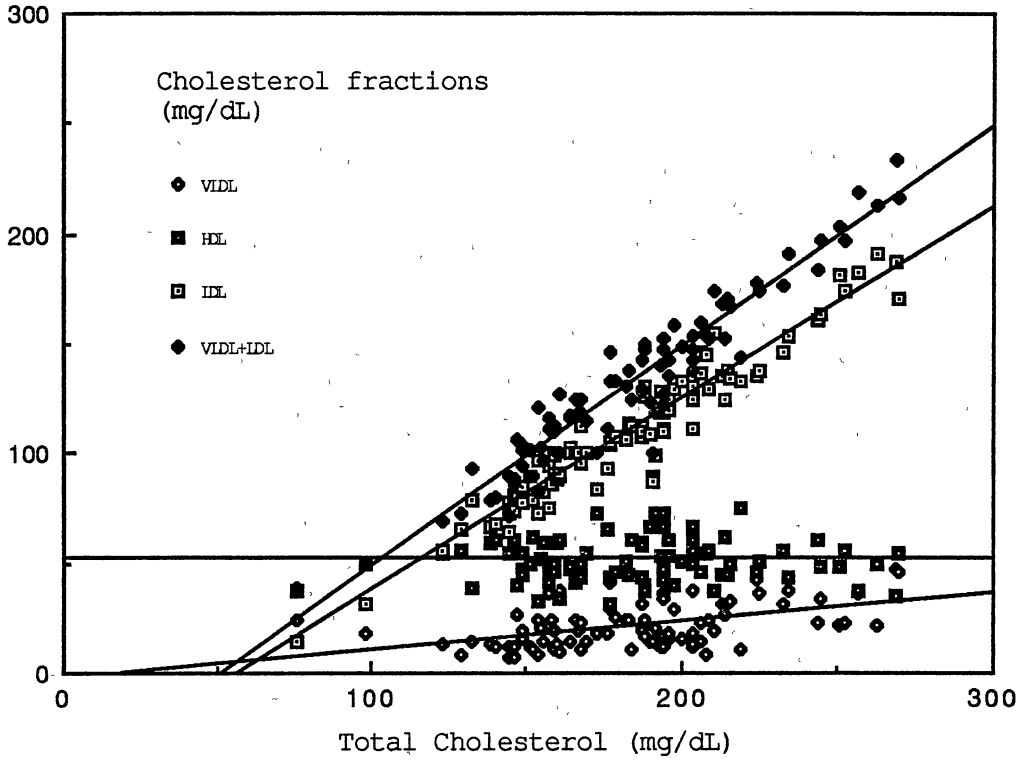


Figure 11. TC vs. HDL-C (a), VLDL-C (b), LDL-C (c), and (VLDL+LDL)-C (d) for Laboratory (D). Correlation equations are:

(a) $y = 51.6 + 0.002x$ (R^2=0.0);

(b) $y = -2.4 + 0.13x$ (R^2=0.247);

(c) $y = -49.9 + 0.87x$ (R^2=0.93); and

(d) $y = -51.6 + 0.99x$ (R^2=0.922) respectively.

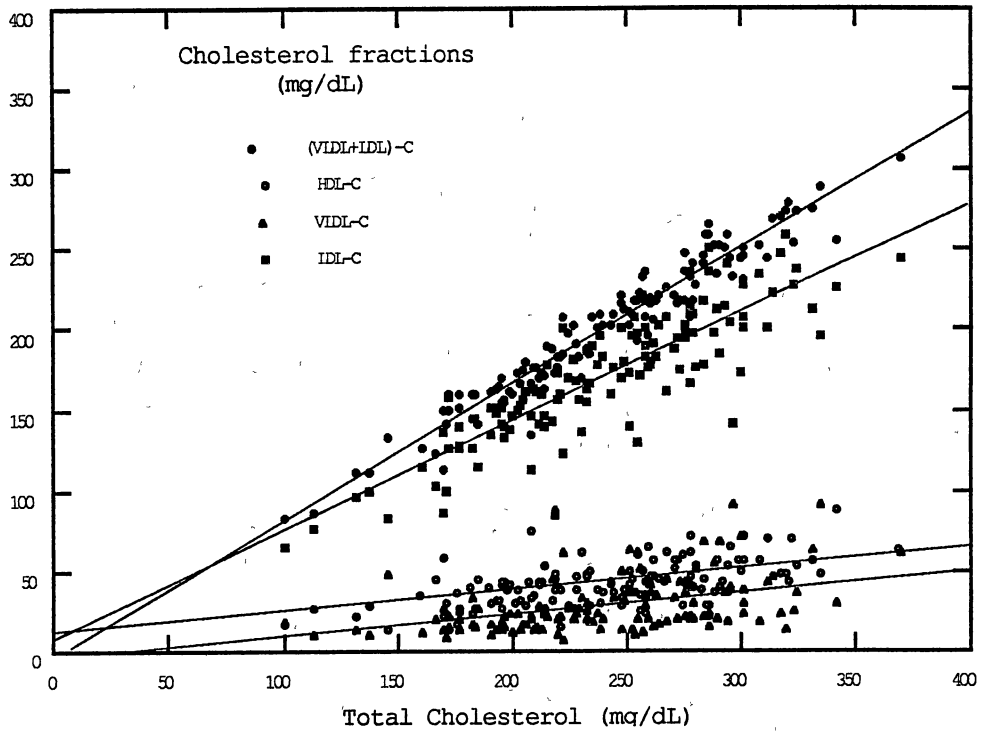


Figure 12. TC vs. (VLDL+LDL)-C, HDL-C, VLDL-C, LDL-C, and for CD Laboratory. Correlation equations are:

(a) $y = -3.594 + 0.8432x$ ($R = 0{,}9702$);

(b) $y = 3.58 + 0.157x$ ($R = 0.5998$);

(c) $y = -9.203 + 0.1582x$ ($R = 0.4568$); and

(d) $y = 5.906 + 0.6826x$ ($R = 0.865$) respectively.

CHAPTER VI

SUMMARY AND CONCLUSIONS

It was the goal of this project to develop a method to determine cholesterol levels which would allow direct determination of high, low and very low density fractions of serum cholesterol. Although this goal was not completely realized the method developed did make it possible to determine HDL-C and the combined VLDL-C and LDL-C fractions directly with a significant increase in precision over currently used enzymatic methods.

The two enzymatic methods and the CD detection method all show good correspondence among the TC values determined. With the exception of the (A) and (D) determinations for the HDL-C fraction all lipid distributions exhibited linear dependence on TC. Measurements of HDL-C made by the commercial enzymatic methods were comparable. The existence of a basic systematic error in determinations of HDL-C made using these conventional methods is a logical assumption. The necessity of a precipitation reaction is one possible cause of this error, since precipitation reactions are difficult to reproduce consistently. Many other factors which could be contributing to the error have been considered (5), one of which is the inconsistencies resulting from the use of different precipitating agents.

It was anticipated that larger errors would result, if methods of determination which had produced large relative inaccuracies (CV>±5%) for the measurement of TC, were also used for the determination of the significantly smaller amounts of HDL-C. With the added difficulty of extracting the low density fractions (5), it was certainly expected that the HDL-C fraction would involve greater experimental error in its measurement than in the measurement of TC. Even with this expectation the correlation of zero was a surprising

result. It was expected that the ratio, TC/HDL-C, lacked diagnostic value due to the errors associated with the HDL-C measurement. It is increasingly apparent that a more accurate and reliable method for the direct determination of HDL-C or LDL-C is a necessity.

The newly developed method combining chromogenic reaction with CD detection exhibits good linearity for correlations between TC measurements and determinations of both HDL-C and (VLDL+LDL)-C. The differences between CD correlations and the (A) and (D) correlations apparently must be due to the errors in the HDL-C measurements obtained from the enzymatic methods since all three methods are in close agreement for TC measurements. It is conceivable that the significant improvement in quality of the HDL-C measurements when the CD detection method is used results from the lack of a precipitation step in these determinations and the fact that CD-inactive substances,such as hemolyzed red blood cells, and high triglyceride levels do not interfere with CD detection. Reconsideration of a second diagnostic parameter, based on the proportion of HDL-C in the TC, may be justified given this improvement in HDL-C data. The precision in (VLDL+LDL)-C measurements have also been greatly improved which may lead to this quantity being a more reliable parameter for reduction therapy monitoring.

It can be concluded from this study that the present NCEP recommendation that patient risk be evaluated only upon measurements of total cholesterol is justified and that as long as measurement of HDL-C is unreliable and inaccurate it has little diagnostic value. The CD detection method discussed here holds promise for accurate measurement of lipid distributions and, therefore should be pursued as a tool for the health industry.

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